DNA repair systems

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Abstract

This paper provides detailed insight into the mechanisms of repair of different types of DNA damage and the direct molecular players (enzymes repairing the damage or tagging the damaged site for further processing; damage sensor molecules; other signalling and effector molecules). The genetic bases of diseases and conditions associated with defective DNA repair are comprehensively reviewed, from the 'classic' severe diseases such as xeroderma pigmentosum and Cockayne syndrome to the much more subtle UV sensitivity syndromes. The review analyses the basic molecular mechanisms underlying the relatively rare monogenic diseases of DNA repair and management of genome integrity as well as the common multifactorial diseases and conditions with late onset that are associated with increased levels of oxidative stress (metabolic syndrome, diabetes type 2, cardiovascular disease) and with accumulation of 'errors' in DNA (normal and pathological ageing phenotypes, various cancers). The role of cell cycle checkpoints in dividing cells and the mechanisms of decision-making for the fate of a damaged cell are discussed with regards to the cell homeostasis in normal and cancerous tissues. The role of major DNA damage-associated signalling and effector molecules (p53, ATM, poly-(ADP-ribose)-polymerase, DNA-dependent protein kinase, BRCA proteins, retinoblastoma protein, and others) is discussed and illustrated with examples in the context of health and disease. DNA repair and programmed cell death are viewed together as a unified mechanism for limiting the presence of damaged cells and cells with potentially oncogenic transformation in multicellular organisms. Special attention is paid to ageing as a natural phenomenon and an adaptive evolutionary mechanism, with a brief outline of 'successful ageing'. The differential rates of repair of DNA in transcribed and nontranscribed regions of the genome and the specificities of DNA repair profile in some types of cells (terminally differentiated cells, pluripotent stem cells, etc.) and in certain taxonomic groups (e.g. 'the rodent repairadox') are discussed with regards to replicative ageing and the evolutionary processes on micro- and macroscale. The role of mutagenesis as a 'hit and miss' mechanism and the 'leakiness' of DNA repair for increasing genetic diversity in the course of individual life and on evolutionary scale and the phenomenology of ongoing molecular evolution are extensively reviewed.

Citation: Chakarov S, Petkova R, Russev GCh. DNA repair systems. Biodiscovery 2014; 13: 2; DOI: 10.7750/BioDiscovery.2014.13.2

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Received: July 8, 2014; **Accepted:** September 18, 2014; **Available online /Published:** September 22, 2014
Keywords: DNA repair, carcinogenesis
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Conflict of Interests: No potential conflict of interest was disclosed by any of the authors.

1. Types of DNA repair systems

If you wish for peace, prepare for war.
Publius Flavius Vegetius Renatus, De Re Militari
(circa 380 AD).

DNA damage is a very broad term, encompassing many different types of lesions in DNA. Accordingly, DNA repair comprises many different types of pathways and mechanisms covering virtually every possible type of injury to the sequence or the structure of DNA. Accepting Lewin's definition of DNA damage "*... any change that introduces a deviation from the usual double-helical structure*" [1] , we may present the following broad definition of DNA repair:
"*Any pathway, process or mechanism that results in restoration of the usual double-helical structure and the biological functions of the damaged DNA molecule*".
Several principally different strategies for DNA repair are currently known to exist [2–4]. Every different strategy is associated with repair of different types of damage and, respectively, with specific mechanisms, molecules and pathways. Sometimes, different pathways may eventually converge, especially in the early stages, as the same signalling molecules may play a role in different types of repair.
The basic types of DNA repair strategies may be listed as follows:

- **Direct repair**
  This is applicable to localised lesions where the effect can be reversed in an energy-dependent process (e.g. repair of photoproducts in DNA by photoreactivation).
- **Removal of the damaged DNA region** (base excision repair (BER), mismatch repair (MMR)) or the region surrounding the damage site (nucleotide excision repair, NER). This strategy for repair encompasses several different mechanisms for repair of different types of damage – modified bases (BER), mismatched bases (MMR), dimers and adducts in DNA (NER), etc. Repair by removal of the damaged DNA region may rely on very different mechanisms, but there are some unifying features – the damaged region (regardless of its size, e.g. a single nucleotide or a DNA fragment) is removed from the molecule and the missing DNA is resynthesised, using the complementary strand of the same DNA molecule as a template for resynthesis. The latter may become an issue in repair of DNA mismatches, as unless there is a specific mechanism for recognising which strand is the 'original' and which carries a

mismatch, the repair may actually cause a mutation to become fixed in DNA and transmitted to the progeny. To avoid this, designated mechanisms exist that make the 'original' strand and the newly synthesised strand distinguishable from one another within a limited time interval (see below).

- **Repair of strand breaks**

  Repair of single-strand breaks (SSB) is pretty straightforward, though slight variances may exist with regard to the type of ends that need to be joined (see below). Management of double-strand breaks (DSBs) is another matter, as more than a couple of DSBs per genome may seriously compromise its integrity. Presence of DSBs in DNA is strictly controlled in living cells, justifying the need for special mechanisms for repair of double-strand breaks. Repair of DSBs is carried out by homologous recombination or non-homologous end joining mechanism. The template for synthesis is provided by a different DNA molecule, or a different region of the same DNA molecule.

Below we present brief descriptions of the basic types of DNA repair.

1.1. Direct repair

This is a mechanism used for repair of alterations in DNA structure that may be reversed directly to the state they were before the damage has occurred. Direct repair works precisely on the damage site and may be employed only for reversal reactions that are thermodynamically possible.

Direct repair is an exclusively prokaryotic repair mechanism. As prokaryotic genomes are relatively short, very dense with genes and exist in one copy only, every instance of damage to DNA may harm the cell and/or eventually cause its death. Therefore, direct repair is an extra defence mechanism in prokaryotes, usually targeted against the most common and deadly, but, at the same time, hardy avoidable environmental damaging agent – the UV light. There are exceptions, of course. For example, O6-methylguanine DNA methyltransferase works on alkylated nucleotides. Direct repair restores the initial nucleotides from UV-induced photoproducts by cleaving the newly formed chemical bond/s (e.g. the 6'-4' bond in 6'-4' photoproducts, and the 5'-5'/6'-6' bonds in cyclobutane pyrimidine dimers). The process requires the presence of light from the visible spectrum (providing the energy to cleave a covalent bond) and involves electron transfer [5,6]. Typical direct repair mechanisms are, for example, photoreactivation and repair by T4endonuclease V.

In purely theoretical sense, ligation of single-strand breaks producing a free 3'-OH end and a phosphorylated 5'-end may also be considered a mechanism for direct DNA repair. In this case, however, the cellular machinery for repair would not even be primed and assembled, as the damage is normally repaired by virtue of simple ligase activity.

1.2. Repair by removal of the damaged DNA region

Depending on the size of the affected region, this is base excision repair or mismatch repair (when the damage affects a single nucleotide) or nucleotide excision repair (when a larger fragment of DNA is removed and subsequently resynthesised). The size of the damaged

region does not always correlate with the size of the region removed in repair. In BER, for example, one nucleotide is affected and, correspondingly, one nucleotide is removed. In NER, however, several dozens of nucleotides are normally removed. In MMR, the size of the removed region may vary from 1 nucleotide to up to 1 Kb of DNA.

Excision repair (ER) [7,8] is a universal mechanism for repair of damaged DNA. It exists in all living organisms, prokaryotic and eukaryotic alike. In mammals, inborn deficiencies of excision repair may present as monogenic or, as was more recently discovered, as multifactorial inherited disease with or without a phenotype of increased cancer proneness. ER is based on removal of a damaged nucleotide or a stretch of several nucleotides containing the damage site from one of the strands in DNA and de novo DNA synthesis using the complementary chain (which is presumably intact) as a template.

Depending on the size of the excised region, excision repair is subdivided into base excision repair (BER) and nucleotide excision repair (NER). Types of DNA damage repaired by BER and NER may be different. The enzymatic activities and the mechanisms involved are also different.

1.2.1. Base excision repair (BER)

BER protects the cellular genome from modification of nitrogenous bases in DNA. Modifications may be produced by oxidation (reactive oxygen species or other free radicals caused by normal metabolism and/or exogenous agents, e.g. ionising radiation) and base alkylation. About 20,000 endogenous lesions in DNA are repaired by BER per day in an eukaryotic cell [9]. Briefly, the damaged base is recognised and removed by a specific glycosylase, producing an abasic site. The DNA backbone is then cleaved at the abasic site, the remainder of the abasic nucleotide is hydrolysed, releasing a free reactive 3'-OH end. The correct nucleotide is then added to the 3'-OH end of the repaired strand. Finally, the gap is ligated by DNA ligase.

Naturally occurring abasic sites in eukaryotic cells (a very common type of damage) are usually repaired by the general mechanism of BER, without the glycosylase activity. The abasic site is recognised and removed by an AP-endonuclease. Then, the missing nucleotide is resynthesised by DNA polymerase β and the gap is ligated by DNA ligase (III or I) [10,11].

1.2.2. Nucleotide excision repair(NER)

Repair by excision of nucleotides works by removing whole stretches of single-strand DNA encompassing the damage site from both sides. Then the missing polynucleotide sequence is resynthesised using the intact strand as a template and the chain is ligated. NER is the repair mechanism with the highest degree of versatility, as it can process virtually all types of damage, including these that could be repaired by direct repair, BER and MMR. As commonly occurring types of damage are often repaired by designated repair pathways (photolyase and related activities for direct repair of photoproducts, BER for oxidised or alkylated bases, base mismatch pathway for mismatched bases, etc.), NER usually repairs bulky adducts in DNA and, in organisms that do not employ photoreactivation, it also deals with photoproducts in DNA.

1.3. Mismatch repair (MMR)

MMR is activated when mismatched bases (that is, base pairs which do not conform to the classic A:T, G:C pairing rule) are present in DNA. Base mismatching occurs normally during

DNA copying, due to the natural error-proneness of the mechanisms of DNA biosynthesis and the mechanisms of processing of intermediates in DNA recombination. A mismatch in DNA is considered a structural type of damage, as it distorts the normal double-helical structure. It may, however, change the information coded by the DNA as well. A mismatch may become a transmissible mutation upon the next cycle of replication, as the newly synthesised daughter strands would 'accommodate' the mismatch – that is, they would add a matching nucleotide against the nucleotide of the mismatch, as there is no mechanism in replication to aid in distinguishing the 'original' sequence. This would effectively lead to producing one double-strand that is identical to the strand as it was before the mismatch occurred and one mutant double-strand, carrying the mismatched nucleotide matched to a complementary nucleotide. The original and the mutant strand would be distributed into identical daughter cells, which would generally have an even chance of producing progeny. Generally, the mismatch must be promptly recognised and repaired before the next cycle of replication, in order to avoid creation of mutant clones. MMR is a complicated mechanism even in prokaryotes, requiring many proteins, acting in scanning the DNA for mismatches, unwinding the surrounding DNA structure and cleaving the DNA backbone in the vicinity of the site of damage, generating a free reactive 3'-OH end. Finally, as in all types of repair, the missing fragment is resynthesised and the 3'-OH end is ligated to the adjacent 5'-phosphate.

1.4. Repair of breaks in DNA

1.4.1. Repair of single-strand breaks

Single-strand breaks producing a free 3'-OH end and a phosphorylated 5'-end are repaired by simple ligation. When, however, the break results in a 5'-OH end and a 3'-phosphoribosyl end, the ends need to be processed before the actual repair is carried out. Designated enzymatic activities (e.g. polynucleotide kinase/phosphatase (PNK), containing both 5'-kinase and 3'-phosphatase activities) may be employed to produce 3'-OH and 5'-phosphate ends which may then be joined [12]. In case there is missing DNA between the restored ends, single-strand DNA synthesis is initiated from the free 3'-OH end, then the 3'-OH end of the newly synthesised DNA is ligated to the adjacent nucleotide in the repaired strand. XRCC1 protein, a stabilising factor for DNA ligase III is a component of the single-strand break repair complex together with PNK and DNA polymerase beta [13].

1.4.2. Repair of double-strand breaks

DSBs in DNA present the most ostensible treat to genome integrity than any other type of damage. Unrepaired or incorrectly repaired DSBs may lead to chromosome breakage and fusion, translocations, aneuploidies, etc.

The basic repair strategy of double-strand breaks is based on molecular recognition between single DNA strands. Repair may occur by homologous recombination or by non-homologous end joining. HR requires the presence of shared regions of homology between the recombining molecules or between similar DNA sequences, existing in more than one copy in the same DNA molecule. In some cases even only several nucleotides may suffice for a region of homology, but usually the homologous stretches used in HR are longer. Unlike homologous recombination, NHEJ does not require similarity between the two interacting DNA molecules. Indeed, NHEJ may make use of regions of micro-similarity (2–4

bp long) between the two strands but it can also join double-stranded ends that are not complementary (Ku-dependent NHEJ) [14].

1.5. Translesion replication

DNA repair is not always unconditionally associated with restoration of the original DNA sequence (that is, the way it was before the damage occurred). In some cases when the damage to DNA is too severe or chronic, the activation of an emergency response mechanism may occur, allowing for bypass replication of damaged templates. This is, essentially, the cell's last resort when survival and/or continued division is deemed crucial, despite the fact that the DNA is seriously damaged. Since the probability of adding an 'incorrect' nucleotide is naturally higher than adding the only 'correct' nucleotide, translesion replication is always associated with risk of introduction of mutations.

Replication is generally stalled at damaged templates until the damage is repaired or else the cell is routed to the apoptosis pathway. Translesion response allows replication of damaged DNA templates using low-fidelity DNA polymerases which do not stop at the damage site but, rather, bypass the damage adding to the growing strand nucleotides which may or may not comply with the base pairing rule. These are polymerases with relatively high error rate, which may incorporate either the correct nucleotide or any other of the four, which may depend on the type of the damage in the template. In cases of some types of damage the translesion polymerases may be actually very precise. For example, polymerase h (eta) replicates with high fidelity DNA with pyrimidine dimers and/or 8-oxoguanine, adding two adenines in the growing strand against the site in the template containing thymine dimers and a cytosine against the 8-oxoguanine in the template strand, but may be less accurate when copying templates containing other types of damage [15, 16]. The variant form of xeroderma pigmen¬tosum (XP-V) is caused by defects in the human gene coding for polymerase eta [17].

Translesion DNA synthesis is believed to be an adaptive mechanism, developed in the process of evolution to ensure a last remedy in cases when the survival of the cell is at stake.

Evidently, the basic types of damage repair are partially overlapping with regard to their substrates, that is, almost every type of damage in DNA may be repaired by at least one (usually more than one) repair mechanisms. Nevertheless, every living creature on Earth, regardless of its size and complexity, is equipped, at the very least, with a system for repair of mismatched bases, a system for repair by excision (BER and/or NER) and a system for repair by recombination/NHEJ. The specificities in every type of repair, however, may be subject to significant variation in different groups of organisms. For example, nucleotide excision repair exists in virtually all types of organisms, but the properties of the proteins carrying out the repair activities may be somewhat different between species.

Some types of DNA repair may be active only in certain types of organisms. For example, the mechanism of direct repair by photoreactivation is typically seen in prokaryotes, lower eukaryotes (e.g. yeast) and, sometimes, in plants. The soya bean plant (Glycine max) repairs photoproducts in its DNA by photoreactivation rather than by NER [18, 19]. NER is a universal mechanism for all living creatures, but eukaryotic mitochondria rely predominantly on BER to repair damage to their DNA, with NER being virtually non-existent

in mitochondria [20, 21]. As the major damage to mitochondrial DNA is base oxidation, which is repaired by the mechanism of BER, it is possible that the use of NER to repair mitochondrial DNA was lost adaptively in the course of evolution.

Finally, the efficiency of certain repair mechanisms may be different between different groups of organisms and even within different cell types in the same organism. For example, in rodents the efficiency of repair of transcribed genomic regions may strongly prevail over the efficiency of repair of untranscribed regions. Repair of untranscribed regions may be specifically inhibited in certain types of terminally differentiated cells (e.g. adult neurons) [22, 23]. All these differences have presumably developed in the course of evolution as specific adaptive mechanisms, equipping the cells and the organisms with diverse set of tools for management of genome integrity.

2. Enzymatic activities of DNA repair

2.1. Enzymology of direct repair

2.1.1. Photolyases

Direct repair of photoproducts in DNA is carried out by several enzymatic activities united under the umbrella term of photolyases (deoxyribocyclobutadipyrimidine pyrimidine-lyases, EC 4.1.99.3) [2, 24]. Depending on the type of the chromophore, photolyases are subdivided into class I (containing a core made of 1,5-dihydroFAD and a pterin derivative (5,10-methenyltetra-hydropteroylpolyglutamate) and class II (containing a 8-hydroxy-5-deazaflavin core) [25]. The photolyases of E. coli and yeast, for example, belong to class I, while the photolyase of Streptomyces griseus is class II. Depending on their substrate specificity, different photolyases may be divided into three types: deoxyribodipyrimidine-photolyase (EC 4.1.99.3); (6-4)-DNA-photolyase (EC 4.1.99.13) and spore photoproduct-photolyase (EC 4.1.99.14).

UV-photolyase activity is typical of prokaryotes resistant to UV, such as M. luteus; some extremophiles (Halobacterium spp., Thermus spp., and others); some species of algae (e.g. Anacystis nidulans) and yeast [26–29]. Therefore, it came as a surprise when an enzymatic activity close to the photolyase activity was identified in virtually all tissues of mammals, including deep-seated tissues in which the access of UV light was virtually impossible [30]. Afterwards, it has been demonstrated that in higher eukaryotic organisms the enzymatic complexes related to photolyases(usually termed 'cryptochromes') were involved with the maintenance of circadian rhythms rather than with DNA repair [31–33]. The ancient function of cryptochromes in repair is not completely abandoned, however, as it has been shown that the presence of DNA damage (e.g. inflicted by UV) may reset the circadian clock in lower eukaryotes(yeast, protozoa) [34–37]. Damage-dependent circadian clock reset was later observed in cultured mouse cells and in vivo in rats subjected to traumatic brain injury [38, 39].

2.1.2. T4 endonuclease V

T4 endonuclease V(T4 endoV nuclease, T4endoV, pyrimidine dimer glycosylase, EC 3.1.25.1) is a dual-activity enzyme coded by the *denV* gene of the T4 phage. T4endoV actively scans DNA for the presence of dimers, binds to them and employs its glycosylase activity in order

to cleave the N-glycoside bond in 5'-pyrimidine of the dimer, creating an abasic site. Then, it uses its lyase activity to hydrolyse the phosphodiester bond immediately before the resulting abasic site, creating a single-strand break[40, 41].

The mechanism of DNA repair associated with T4 endonuclease V is not, strictly speaking, resulting directly in restoration of the initial DNA structure. Rather, it is a mechanism of activation of DNA repair by generating a recruitment signal for the repair machinery to assemble at the damage site, as the presence of single-strand breaks in DNA is a more powerful signal for recruitment of the repair machinery than the presence of dimers.

2.1.3. Other types of direct DNA repair

Direct DNA repair in prokaryotic organisms may be associated with other enzymatic activities as well. Among these are the already mentioned spore photolyase (SPL) and O6-methylguanine DNA methyltransferase (ada, ogt). Spore photolyase deals with UV-induced adducts in DNA that are generally unique to spores (hence the need for a specific mechanism). Unlike most mechanisms of direct repair, O6-methylguanine DNA methyltransferase (MGMT, EC 2.1.1.63) repairs damage inflicted by alkylation (usually, methylation) of bases. The enzyme catalyses the restoration of the original nucleotide by transferring the alkyl radical onto its own molecule (self-alkylation) [42]. More details about the exact process may be found in Chapter VI - Mechanisms of DNA repair.

2.2. Enzymology of excision repair

2.2.1. Enzymatic activities of base excision repair

The major enzymatic activities involved in repair by excision of bases are DNA-glycosylases, apurine/apyrimidine endonuclease/lyases, DNA polymerases and DNA ligases. The latter two enzymatic activities are common for all excision repair pathways, as the recovery of the DNA from the excised region is invariably by DNA synthesis, which leaves behind a gap subject to repair by ligation. Five different genes coding for DNA glycosylases have been identified in E. coli [24]. In higher animals, all genes coding for the eukaryotic homologues of prokaryotic N-glycosylases have been identified, plus several more that are only seen in mammalian cells [43–45].

DNA glycosylases recognise the damaged base and cleave the N-glycoside bond between it and the attached deoxyribosyl residue, creating an abasic site (apurinic or apyrimidinic site, AP-site). The AP-site is subsequently processed by AP-lyase (EC 4.2.99.18). There are 4 basic classes of AP lyases. Class I and class II AP-lyases break C-O-P bonds producing 3´-OH and 5´-phosphate termini. Class III and class IV lyases cleave C-O-P bonds generating a 3´-phosphate and a 5´-OH end [46, 47]. The3'-phosphate is subsequently removed by AP-endonuclease or, in mammals, by polynucleotide kinase/phosphatase, which may also phosphorylate the 5'-OH [47, 48]. The missing nucleotide is added to the 3'-OH end by DNA polymerase and the single-strand break is repaired by DNA ligase.

Many types of DNA glycosylases exist, varying significantly in their substrate specificity. BER glycosylases are generally classed as monofunctional or bifunctional. The former possess glycosylase activity only, while the latter also exhibit lyase activity. In fact, the majority of the bifunctional endonucleases are officially classed as lyases by NC-IUBMB (Nomenclature Committee of the International Union of Biochemistry and Molecular Biology).

Occasionally, sites in DNA where there is no damage present may be subjected to repair, or, to be precise, to excision of a fragment of DNA and subsequent resynthesis. This occurs most often in BER, as glycosylases excising modified nucleotides may non-specifically excise an additional number of undamaged bases [49].

Depending on the type of substrate they act upon, BER-glycosylases may be subdivided into three distinct types:

Uracil glycosylases – removing uracil from DNA

Uracil may be present in DNA because it was incorporated by the DNA polymerase by mistake or it may have resulted from spontaneous deamination of methylated cytosine. Prokaryotic uracil DNA glycosylase (Udg) and its mammalian homologues (Ung and, respectively, UNG in humans – Table I)are very similar in terms of amino acid content and structure [50]. Some authors believe that the emergence of the mechanism repair of nucleic acids early in evolution was one of the drives for the almost complete exchange of RNA for DNA as the carrier of the genetic information. The reasoning behind this is that while uracil-glycosylase promptly recognises and removes uracil from DNA (where it ought not be present at all) but in RNA it could hardly distinguish uracil resulting from spontaneous deamination of methylcytosine from the normally occurring uracil. As methylcytosine deamination is a very common event, occurring dozens of times per day in the cell, in a RNA-based world, UDG would have been constantly at work, causing needless processing of uracil present where it actually belongs and needlessly delaying the process of copying of the genetic information (as a template currently under repair cannot be copied until the repair activities are complete).

Glycosylases processing alkylated bases

These are usually monofunctional enzymes, recognising and excising alkylated (most commonly, methylated) bases. Bacteria usually employ two basic N-glycosylase activities for repair of alkylated bases: Tag (3-methylpurine DNA glycosylase I) and AlkA (Table I). The Tag activity is specific to the most common cytotoxic products of methylation – 3-methyladenine and 3-methylguanine [51]. Eukaryotic homologues of 3-methylpurine glycosylase are, for example, yeast 3-methyladenine glycosylase Mag1and human N-methylpurine DNA glycosylase (MPG) [52, 53]. AlkA constitutes roughly 10% of the N-glycosylase activity in prokaryotic cells[54, 55]. The AlkA protein takes part in the adaptive response of the cell to alkylation and it transcription is induced when prokaryotic cells are treated with high doses of alkylating agents. A homologous activity has not been identified in humans so far.

Often the specialised literature mentions another N-glycosylase activity alongside with AlkA, namely, AlkB (alpha-ketoglutarate-dependent dioxygenase, EC 1.11.4.-), despite the fact that AlkB does not possess glycosylase activity at all. AlkB is specific to alkylated bases such as 1,N6-ethenoadenine, 3,N4-ethenocytosine, etc., but it works by a different mechanism[56]. It catalyses decarboxylation of a-ketoglutarate and hydroxylation of alkylated bases in a coupled reaction, resulting in the restoration of the normal base and release of formaldehyde[57].

Glycosylases processing oxidised bases

These recognise and process products of oxidative stress, e.g. 8-oxoguanine;N3-methyladenine; N7-alkylguanine; 2,6-diamino-4-hydroxy-5-formamidopyrimi-dine and other purine and pyrimidine derivatives. Two major types of BER glycosylases recognising oxidised bases have been identified in bacteria – formamidopyrimidine DNA glycosylase (Fpg) and MutY (Table I). Homologous enzymatic activities have been identified in eukaryotes. Bacterial Fpg removes formamidopyrimidines, 8-oxoguanine and other oxidation products from DNA[58, 59]. The human homologue of FPG is called hOGG1. Somatic mutations in the *hOGG1* gene have been identified in some tumours and inherited polymorphisms may be associated with increased risk for genetic disease [60, 61]. MutY (A/G-specific adenine glycosylase) is a DNA glycosylase which excises A specifically from mismatched G-A pairs. The efficiency of removal of A is enhanced when the mispaired nucleotide is 8-oxoguanine [62]. An additional enzymatic activity assists in protection of the cell from oxidative stress – MutT, a deoxy-GTP-ase (EC 3.6.1.-) that inhibits the incorporation of 8-oxoguanine during replication. Enzymatic activities similar to MutY and MutH (MUTYH, A/G-specific adenine DNA glycosylase) have also been identified in man [63–65]. One of the hereditary cancer syndromes in man is associated with carriership of mutations in the *MUTYH* gene.

Table I lists the major prokaryotic N-glycosylases and presents a comparison between with the N-glycosylases identified so far in man.

Table 1. Bacterial and human homologues of N-glycosylases of base excision repair.

Proka-ryotes (E. coli)	Man	Functionality	Substrate	Classification by NC-IUBMB
Udg	UNG	Monofunctional	Uracil	3.2.2.27 3.2.2.28
AlkA	Not identified	Monofunctional	Alkylated bases	3.2.2.21
Tag	MPG	Monofunctional	Alkylated bases	3.2.2.20
Fpg	OGG1	Bifunctional	Oxidised bases	3.2.2.23
MutY	MUTYH	Monofunctional	Oxidised (e.g. 2-hydroxy-adenine) and mispaired bases – e.g. adenine from A:G and A: oxoguanine mispairs	3.2.2.-

An example of mammalian-specific glycosylase is G/T thymidine glycosylase (TDG, EC 3.2.2.29). It catalyses the removal of T from mispaired G/T pairs and C/T and T/T mispairs [66]. The G/T mispair correction, however, is carried out with higher efficiency than the latter, probably because of the high frequency with which the C/T conversion (by 5-methylcytosine deamination) normally occurs.

Apurinic/apyrimidinic (AP)-nucleases and AP-lyases

These two enzymatic activities work together to ensure that the apurine/apyrimidine (AP-) sites generated by the action of the BER glycosylases are properly processed so that no abasic sites persist in DNA. Three basic AP-endonuclease activities were initially identified in bacteria, named Nth (endonuclease III), Nei (endonuclease VIII) and Nfo (endonuclease IV, EC 3.1.21.2), respectively[67]. Nth and Nei were initially catalogued as bifunctional endonucleases (that is, possessing both endonuclease and AP-lyase activities). The later versions of the NC-IUBMB, however, questioned the nuclease activity of Nth and Nei, as

they cleaved the phosphodiester bond in DNA not by hydrolysis, but by beta-elimination, and they were eventually moved to the lyases chapter of NC-IUBMB [68]. Currently, Nth and Nei and are both referred to by NC-IUBMB as a single AP-lyase activity (4.1.99.18) [69, 70]. The respective eukaryotic homologues of Nth and Nei are Nth1 and Neil. The human homologue of Nth, NHTL-1 (hNth-1) acts to excise oxidised pyrimidine derivatives [71, 72]. The Nei homologues in man are more than one, and are named NEIL1, NEIL2 andNEIL3, respectively[73, 74]. NEIL1 excises oxidised pyrimidine derivatives from DNA– preferentially thymine glycol, 5-hydroxycytosine, dihydrothymine and, with lower affinity – dihydrouracil and 5-hydroxyuracil. The substrate specificity of NEIL1 to oxidised purines, e.g. 8-oxoguanine is relatively low [73]. Both NEIL1 and NEIL2 recognise and remove spiroiminodihydantoin and guanidinohydantoin from DNA [75]. The pre-mRNA of NEIL1 is subject to RNA editing in human cells, causing a lysine-to-arginine substitution in the lesion recognition loop of the protein [76]. The two resulting forms of NEIL1 exhibited different enzymatic properties, with the Arg variant removing thymine glycol from DNA at a slower rate than the Lys variant, whereas the Arg variant enhanced the removal of guanidinohydantoin from DNA.

NEIL2 catalyses the excision of oxidative products of cytosine, specifically 5-hydroxyuracil, but also 5,6-dihydrouracil and 5-hydroxycytosine[77]. NEIL3 is a DNA glycosylase with a somewhat different substrate specificity and unusual structural features compared to other Nei homologues. NEIL3 removes oxidised purine nucleosides from DNA (guanosine derivatives – spiroiminodihydantoin, guanidinohydantoin, and also 5-hydroxy-2'-deoxycytidine, 5-hydroxy-2'-deoxyuridine, albeit with lower efficiency than hydantoin lesions), also 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG), 4,6-diamino-5-formamidopyrimidine (FapyA) and 8-oxoadenine, but not 8-oxoguanine [43, 78]. The biological functions of Neil3 are still not as well studied as the other Nei homologues [79].

Inherited defects in NEIL1 gene have been shown to increase the risk for some multifactorial diseases in man (e.g. diabetes type 2).

Apurinic/apyrimidinic exonuclease activity is another enzymatic activity utilised in BER and also in mismatch repair. Bacteria have a designated apurinic/apyrimidinic exonuclease activity –exonuclease III (Xth), a multifunctional enzyme acting in repair of oxidised bases [80, 81]. Human apurinic/apyrimidinic endonuclease (APEX nuclease, APE1) possesses apurinic/apyrimidinic (AP-) endonuclease activity, but also has 3'-5'exonuclease activity, and DNA 3'-repair diesterase and DNA 3'- phosphatase activities [82–84]. APE1 hydrolyses the phosphodiester bond to the 5'-side of abasic sites generated in BER, resulting in single-strand break with 5'-deoxyribose phosphate and 3'-OH ends. APE1 may also work as an exonuclease on mismatched nucleotides at the 3'-ends of gapped or nicked DNA molecules[84].

DNA polymerases and DNA ligases of BER

The removal of the AP-site leaves behind one nucleotide missing from the DNA strand and a single-strand break. The resynthesis of the missing nucleotide in prokaryotes is usually carried out by DNA polymerase I but polymerases of the X family are also capable of carrying out the synthesis [85, 86]. The X family of polymerases comprises of polymerases beta (β), lambda (λ), mu (μ) and sigma (σ), and terminal deoxynucleotidyl transferase (TDT).

All exhibit structural and functional resemblance to TDT [87]. Unlike 'nominal' polymerases (such as polymerase I of E. coli), which possess DNA-dependent polymerase activity, 3'–5' exonuclease (proofreading) and 5'–3' exonuclease activity, PolX polymerases possess only DNA polymerase and 3'–5' exonuclease activities [88]. In eukaryotes, BER repair polymerases may be somewhat different depending on the species. In yeast, it is DNA polymerase δ, while in mammals it is usually DNA polymerase β that resynthesises the missing nucleotide in BER, though polymerase λ may also be used, when the patch that must be resynthesised is longer than one nucleotide (long-patch BER) [89, 90]. A couple of years ago were identified several mutant variants of the human DNA polymerase β that allow for bypass copying of DNA containing abasic sites [91].Polymerase beta is also responsible for the excision of the 2-deoxyribose-5-phosphate at 5'-apurinic/apyrimidinic sites in short-patch BER [92].

The 5'-flap endonuclease 1 (FEN1, DNase IV) catalyses the processing of reaction intermediates (5'-flaps) generated when DNA polymerase encounters the 5'- end of a downstream Okazaki fragment [93–95]. FEN1 is an evolutionarily conserved component of DNA replication an almost all living organisms, prokaryotic and eukaryotic alike[96]. The yeast homologue is called Rad27 [97]. High degree of homology was observed between Rad27/FEN1 and the 5'-nuclease domain of E. coli polymerase I [98]. Fen1 also removes the 5'-overhanging DNA flaps formed as intermediates in the 'long-patch' mechanism of base excision repair. FEN1 is rapidly recruited at apurine/apyrimidine sites resulting from glycosylase activities during BER [99]. No human disease phenotype associated with inherited mutations in FEN1 has been identified yet, presumably because the associated defect would be incompatible with life. Indeed, mouse embryos carrying null alleles for *Fen1* die early in utero[100]. Mouse embryos with mutations in the nuclease domain of FEN1 are carried to term and capable of independent life, but exhibit high levels of spontaneous mutations, autoimmune disorders, cancer-proneness and chronic inflammation [101].

Ligase III is the primary ligase of base excision repair, although ligase I (which is the primary ligation activity in the replication of the lagging strand) may also function in BER [102]. Ligase I functions in a complex with PCNA(proliferating cell nuclear antigen)[103]. In the nucleus ligase III works as part of a complex with its stabilising factor XRCC1 [82]. In the mitochondria, ligase III functions for the maintenance of the integrity of the mitochondrial DNA independently of XRCC1 [104, 105].

2.2.2. Enzymatic activities of nucleotide excision repair

Over 20 different enzymatic activities are involved in NER. The proteins of NER are assembled at the damage site after induction by specific triggers, in a sequential manner, forming a complicated enzyme complex ('reparosome'), which was initially believed to exist in the cell in a preassembled state. Depending on whether the genomic region undergoing repair is actively transcribed at the moment or not, the triggers for initiation of NER may be different. As a rule, for actively transcribed genes the chief trigger is the presence of stalled RNA polymerase II at the damage site[106]. Some of the subunits of the NER repair complex, such as transcription factor TFIIH, are integral parts of the transcription complex[107, 108]. For untranscribed genes, the damage is recognised and the NER

pathway is started by a specific protein complex, XPC-hHR23B and DNA-damage binding protein (DDB)[109, 110].

After the damage in DNA is recognised as such, the basic steps involve introduction of two single-strand breaks, in 5'- and in 3'- direction from the damage site. It requires 5'- and 3'-endonuclease activities, provided by the proteins ERCC1-XPF and XPG, respectively [111]. The double helix must be unwound in the region of the repair activities, so that the repair machine would gain access to the damage site. This is carried out by two proteins with helicase activity, XPB and XPD. These two are integral parts of the transcription factor TFIIH [107].

The DNA synthesising activity in prokaryotic NER is carried out by DNA polymerase I and II [112, 113]. Polymerase I is the most abundant DNA polymerase in bacterial cells (accounting for about 95% of all the polymerase activities). Besides its role in DNA repair, PolI also acts in the processing of the lagging strand during DNA replication, including the removal of 5'-flaps. In eukaryotic cells, DNA synthesis associated with NER is normally carried out by 'high-fidelity' DNA polymerases – namely, the δ (delta)and ε (epsilon) polymerases[114–117]. Delta and epsilon DNA polymerases are two of the three main polymerases involved in the synthesis of the leading strand in eukaryotic replication.

After the excised fragment is synthesised de novo, the single-strand break is ligated to the remaining part of the repaired strand. In the nucleus, the ligase activity is usually provided by DNA ligase III, complexed with the nuclear factor XRCC1. The latter serves as a stabiliser to ligase III [118]. Eukaryotic cells capable of division may employ DNA ligase I as well [102,116].

2.3. Enzymology of mismatch repair

In prokaryotic cells, the recognition and excision of mismatched nucleotides requires only four specialised proteins – namely, MutS, MutL, MutH and MutU, and also DNA polymerase and DNA ligase [119, 120]. Of these, MutH carries the endonuclease activity and introduces a single-strand break in the strand containing the mismatched nucleotide. MutS dimer recognises the mismatch and binds to the damage site. MutL recruits MutH to the site of the mismatch and activates it [reviewed in 121]. MutU (helicase II, EC 3.6.4.12) unwinds DNA in the vicinity of the mismatch. The synthesis of DNA is carried out by DNA polymerase III holoenzyme [122]. The single-strand break is repaired by DNA ligase [102,122,123].

In eukaryotes, mismatch repair is initiated by MutS homologues (usually, as heterodimers–MSH2-MSH6 (MutS alpha) or MSH2-MSH3 (MutS beta))binding to a mismatched nucleotide pair. MutL alpha is recruited to the heteroduplex after MutS alpha has already bound to the mismatch site. Not all prokaryotes possess the equivalent of MutH endonuclease or a closely related protein homologue. MutH homologues have not been identified in eukaryotic cells yet. Instead, the endonuclease activity is carried by the eukaryotic homologues of MutL – MutLα in yeast and MLH1 in man [124–126].

Eukaryotic homologues of MutU have not been identified yet, but the potential involvement of various eukaryotic DNA helicases in mismatch repair is currently investigated. Among these are, for example, BLM helicase, WRN helicase and the FANCJ helicase [127–129]. BLM and WRN helicases play a role in the maintenance of genome integrity. Inherited deficiencies (Bloom syndrome and Werner syndrome, respectively) are

associated with increased levels of spontaneous mutations. The FANCJ helicase, complexed with BRCA1, acts in repair of double-strand breaks by recombination [130]. The deficiency of FANCJ produces another phenotype, associated with increased genome instability – Fanconi anemia complementation group J.

Yet another enzymatic activity is involved in mismatch repair, namely, exonuclease 1 (Exo1). Exonuclease 1 is a 5'-3' DNA exonuclease specific to double-strand DNA [131]. It also possesses 3'-5' exonuclease activity [132]. Exo1 excises mismatch-containing DNA tracts directed by strand breaks located either 5'- or 3'- to the mismatch. The catalytic activity of mammalian Exo1 is stimulated by an order of magnitude in the presence of a 5'-phosphate group, but is inhibited in the presence of even a single abasic lesion in DNA [133]. Exo1 activity may be directed by strand breaks located either 5'-or 3'- to the mismatch, with MutS-alpha only required in case when the excision is directed by a 5'- strand break, and both MutS-alpha and MutL-alpha needed when the break is located 3'- to the mismatch [134]. Exo1 plays a role in repair of double-strand breaks as well, generating extensive single-strand regions in DNA which subsequently serve as a substrate for homologous pairing and strand exchange [135] (see below).

Eukaryotic MMR makes use of DNA polymerase delta, a replicative polymerase, to synthesise the excised fragment [136,137]. It makes sense, as replicative polymerases exhibit high fidelity, thus making DNA synthesis in mismatch repair as precise as replication. The DNA ligase of MMR in eukaryotic cells is generally ligase I [123].

2.4. Enzymology of repair by recombination

2.4.1. Enzymatic activities of repair by homologous recombination

Homologous recombination is a complicated mechanism of DNA repair, requiring multiple enzyme activities even in prokaryotes. In E. coli the major participants in homologous recombination are the proteins RecA and Rec-BCD which bind to the free ends generated by the DNA break and deploy their exonuclease activities so as to generate a single-strand tail with a reactive 3'-OH end. RecA is also responsible for the maintenance of the free DNA ends in single-stranded state, their invasion into the DNA duplex and the formation of the cruciform structure. Substitutions in single nucleotides do not considerably interfere with the invasion of the reactive free end in the duplex and the migration of the cruciform structure, whereas small deletions or insertions may block the ongoing strand exchange [138].

Helicase activity is required to relax the helical structure around the site of damage. Prokaryotic helicases involved in HR are members of the RecQ family, unwinding double-stranded DNA to yield a single-stranded region in an ATP-dependent process, moving along the length of DNA in a 3'- to 5'-direction [139,140]. The migration of the cruciform structure along the DNA duplex is implemented by the ATP-dependent DNA helicase complex RuvAB [141]. The structure is resolved by the endonuclease RuvC (crossover junction endodeoxyribonuclease, EC 3.1.22.4). RuvC resolves Holliday junction intermediates by introduction of nicks at sites symmetrically opposed at the junction in the homologous arms of the cruciform structure [142,143].

Homologous recombination in eukaryotes exhibits little difference from the prokaryotic mechanism in terms of working principle and basic stages. The initial recognition of the

double-strand break and the processing of DNA ends in eukaryotic cells are carried out by the protein complex MRN (MRE11-RAD50-NBS1).The MRN complex binds with high affinity to broken DNA ends. It possesses DNA unwinding capacity and may tether the broken ends together and/or process them before joining. MRN complex possesses 3'-5' exonuclease as well as endonuclease activities [144]. It may trim (resect) the ends before joining or may excise oligonucleotides from the damaged DNA, thus activating the ATM/ATR system of damage sensing [145,146]. The Rad50 protein is involved in tethering the two broken DNA ends together and facilitating the search for regions of homology for the purposes of recombination [147]. The MRE11 protein carries the 3'-5' exonuclease activity of the MRN complex. This activity is increased when MRE11 is bound to RAD50 [144]. NBS1 (nibrin) acts to recruit and activate ATM, ATR, and probably DNA-dependent protein kinase to the DNA damage site [148].

Unlike bacterial chromosomes, eukaryotic DNA is packed in nucleosomes, therefore, the chromatin structure in the vicinity of DSBs must be rearranged so as to ensure that the repair machinery has unobstructed access to the damage site [149]. The rearrangement relies partly on phosphorylation of histone H2AX, which results in increased negative charge and, respectively, decreased affinity to DNA [150]. Nbs1 plays an important role of recruiting the whole MRN complex to the damaged site, binding to the variant histone H2AX and facilitating the binding of ATM to the complex and its subsequent autophosphorylation [151].

The single-strand binding protein RPA binds to the free reactive DNA ends and facilitates the formation of the cruciform structure.

Three mammalian homologues of bacterial RecQ helicases have been described so far – RECQL2 (WRN), RECQL3 (BLM) and RECQL4. RECQL helicases possess DNA-dependent ATPase, DNA helicase, and 3'-5' single-stranded DNA translocation activities[152,153]. Deficiencies of human HR helicases may produce several disease phenotypes associated with segmental progeria.

The exonuclease Exo1 generates single-stranded DNA stretches that serve as a substrate for RAD51 [135]. The RAD51 family of proteins (eukaryotic orthologues of bacterial RecA) mediates the pairing between homologous sequences and strand exchange [reviewed in 154]. The migration of the cruciform and the subsequent resolution of the recombinant molecules is implemented in eukaryotes by a complex of the proteins XRCC2, XRCC3 and RAD51 [155–157].

The major eukaryotic polymerase acting in the extension of the heteroduplex in repair by homologous recombination is polymerase δ (delta) [158].

Bacterial ATP/ase/helicase RuvAB has two homologues in man, RUVBL1 and RUVBL2, which act as a complex with the product of the protooncogene *c-MYC*[159]. RuvC has one human homologue discovered so far, the Holliday junction resolvase GEN1 [160].

End ligation in repair by homologous recombination is carried out by ligase I or ligase III [161].

2.4.2. Enzymatic activities of repair by non-homologous end joining

NHEJ does not require extensive regions of homology between the interacting molecules. On the contrary, it may modify the ends subject to joining to increase the diversity in the

resulting sequences. The NHEJ machinery in prokaryotes operates on minimal resources – for example, Bacillus subtilis has a minimal NHEJ machinery consisting only of designated DNA ligase (ligD) and the Ku protein, binding to free DNA ends [162,163]. Eukaryotic NHEJ requires more proteins, including the Ku proteins (Ku70, Ku80), DNA ligase IV, its complementing factor XRCC4, the nuclease Artemis (DNA crosslink repair protein 1C) and the auxiliary protein Cernunnos (non-homologous end-joining factor 1). Finally, NHEJ requires the presence of DNA-dependent protein kinase (DNA-PK), a nuclear serine/threonine kinase with signalling functions, which plays a role in the activation of many NHEJ proteins (e.g. XRCC4, the Ku protein components, Artemis, and others) [164, 165]. The Ku proteins function as regulatory subunits of DNA-PK.Ku70 and Ku80 are DNA-dependent ATP-dependent helicases, specific for single-strand DNA [166,167]. The Ku factor is believed to also function in NHEJ as a 5'-deoxyribose-5-phosphate/AP lyase, catalysing the beta-elimination of 5'-deoxyribose-5-phosphate at abasic sites in the vicinity of double-strand breaks [168].

Artemis is a protein, required for the completion of the immunoglobulin class switch recombination (V(D)J recombination) during the maturation of B- and T-lymphocytes in mammals. Purified Artemis possesses 5'-3' exonuclease activity [169]. Upon phosphorylation by DNA-dependent protein kinase, Artemis also acquires endonuclease activity to open the hairpin structures and resect the 3'- and 5'- overhanging tails, generated by the process of V(D)J recombination [169,170]. The resulting free ends are then processed by the error-prone polymerases of NHEJ and subsequently ligated by the XRC4/Ligase IV complex.

The DNA synthesis in NHEJ is carried out by DNA polymerase λ (lambda) or μ (mu), both of the polymerase X family [171]. These polymerases are more error-prone than the polymerases acting in replication and DNA resynthesis in other types of repair [172,173]. Polymerases lambda and mu allow for considerable heterology between the ends that are being joined, as they possess terminal transferase-like activities and are, therefore, capable of creating sequence homology between the free ends(or increasing pre-existing homology) [87]. The ligation of free ends in NHEJ is generally implemented by the XRCC4/ DNA ligase IV complex [174,175].

2.5. Enzymatic activities of translesion template processing

2.5.1. Enzymatic activities of translesion transcription

Translesion transcription may be carried out by normal RNA polymerases, as they are usually capable of adding the 'correct' nucleotide against a site that contains an unusual nucleotide or an abasic site, if this is absolutely necessary. In the presence of some specific modifications, however, e.g. oxidised nucleotides, the transcription is stalled and the TC-NER machinery is recruited.

2.5.2. Enzymatic activities of translesion replication

Bacterial translesion DNA polymerases add nucleotides basically at random, with little regard to base-pairing specificity, but with 100–150-fold efficiency than PolIII would at a damaged template[176,177]. At abasic sites, adenine is the most often added nucleotide, regardless of the pairing partner in the opposite strand [176].

In higher eukaryotes, the error-prone polymerases are seldom activated, as the fidelity of replication is very important. Their activities may serve as a last and desperate attempt to keep the cell cycle going in cells with damaged DNA. Several translesion DNA polymerases have been identified so far in eukaryotes – Rev1 (in yeast); polymerase η (eta), polymerase κ(kappa), polymerase ζ (zeta), polymerase ι (iota), etc. [17,178–182]. Using translesion synthesis, the template integrity is restored, but mutations are introduced at high rate. The risk for introduction of mutations may vary significantly with the type of the error-prone polymerase. Human DNA polymerase eta is a Y-family translesion polymerase that lacks 'proofreading' activity. Its error rate varies widely – one mismatch in 18–380nucleotides, depending on the type of damage and other factors [183]. Unlike most error-prone polymerases, Pol eta processes DNA with thymine dimers with relatively high fidelity, adding two As opposite thymine dimers [184]. In cells in which rapid turnover of cells is important and thymine dimers are among the predominant type of lesions, such as skin cells, polymerase eta may very well manage the replication of the damaged template, as its activity effectively restores the correct DNA sequence in most cases. The possibility for passing on the mutated DNA would be limited anyway, as differentiated keratinocytes carry out most of their functions long after their cell cycling is over (more specifically, after they are already dead). In variant form of xeroderma pigmentosum (XP-V), the underlying defect is in the gene coding for human polymerase eta [17,181]. In individuals with XP-V, the copying of UV-damaged DNA is taken over by the polymerases zeta and iota. The latter also act on damaged templates containing photoproducts, but with lower fidelity than polymerase eta, therefore, an UV-sensitive phenotype eventually develops. Recruitment of error-prone DNA polymerases to sites of DNA damage is dependent on monoubiquitination of the sliding-clamp protein PCNA at a highly conserved lysine residue (Lys164) [185,186]. The Y-family of polymerases, including polymerase eta, possess C-terminal ubiquitin binding motifs, conferring affinity to monoubiquitinated PCNA. Mutations affecting regions of *Rev1* gene coding for these motifs may be associated with loss of function of the encoded protein [187]. The ubiquitination of PCNA is mediated by the Rad6/Rad18 protein complex, where Rad6 is an ubiquitin conjugating enzyme (in man – ubiquitin-conjugating enzyme E2B; UBE2B) and Rad18 – an ubiquitin ligase (in man – RAD18). It has been believed that Rad18 acts upstream of polymerase eta to promote the initiation of translesion synthesis. Recently, it has been reported that Rad18 may targeted to PCNA by DNA polymerase eta itself [188]. Specifically, the authors propose that polymerase eta acts as a non-catalytic effector at sites of stalled replication forks by physically tethering Rad18 to PCNA in order to stimulate PCNA monoubiquitination.

Some of the translesion polymerases, for example, polymerase iota may be overexpressed in epithelial cancers [189]. This may be an adaptive cancer-specific mechanism conferring drug resistance, as polymerase eta was found to be capable of read-through copying of DNA templates containing cisplatin adducts induced by anticancer therapy [190].

3. Mechanisms of DNA repair

3.1. Mechanisms of direct DNA repair

3.1.1. Direct repair by photoreactivation

Repair by photoreactivation works on DNA damage that is thermodynamically reversible, that is, the initial state may be restored if the energy needed for breakage of the de novo formed chemical bonds is available. The source of the energy is usually light from the visible spectrum, a readily and abundantly available source which virtually always accompanies natural UV light (which is the main culprit for occurrence of damage processed by photoreactivation).

Photolyases contain a chromophore core and use $FADH^-$ as a cofactor. The chromophore core absorbs the light from the visible spectrum (300–600nm) and uses the energy to break the newly formed covalent bonds in the photoproducts (usually, cyclobutane pyrimidine dimers) [191,192]. Photolyase activity on a cyclobutane pyrimidine dimer results in restoration of the initial thymines in a process called electron tunnelling [193]. Briefly, the excited $FADH^-$ donates one electron to the dimer. The cyclobutane ring in CPD− breaks, and the electron is returned to FADH•, restoring the damaged DNA and the catalytic cofactor (Fig. 1).

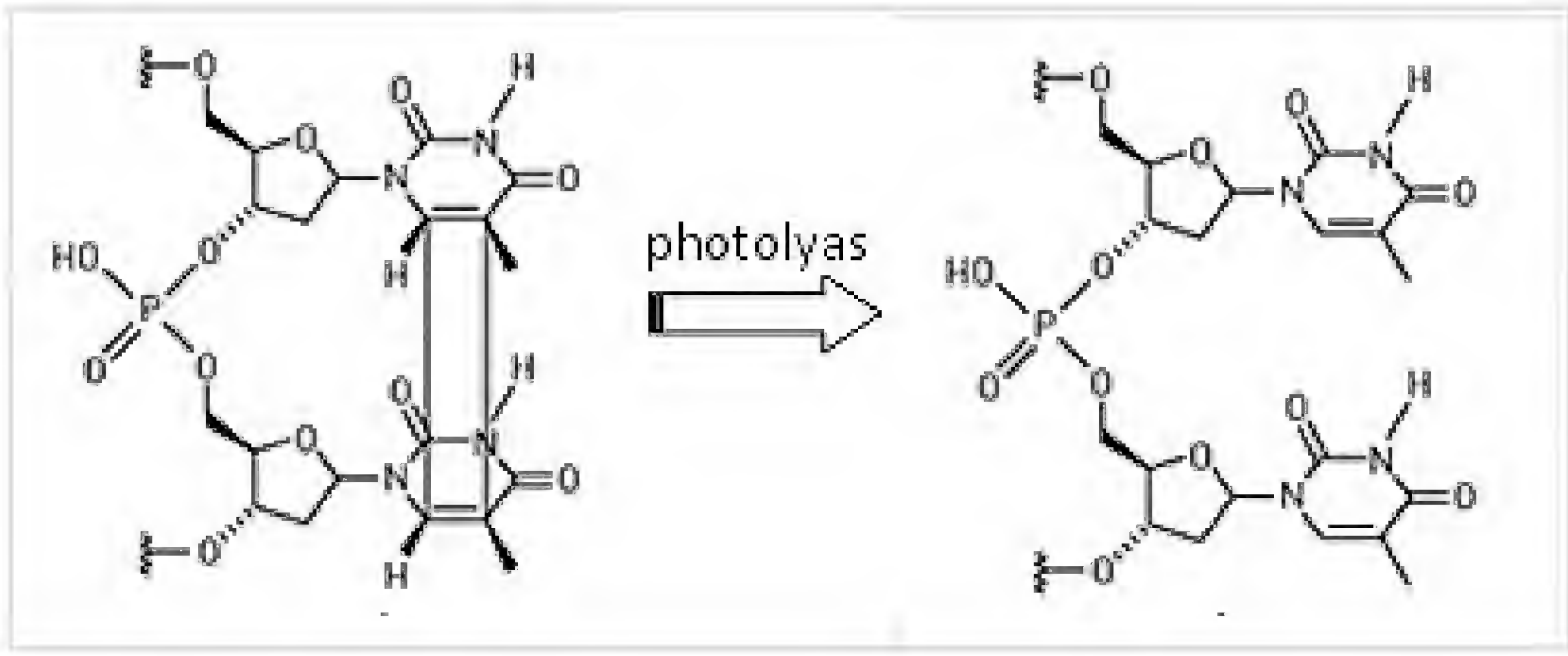

Figure 1. Photolyase-catalysed restoration of initial thymines from a thymine dimer.

Photolyases were initially called "UV-endonucleases". This term, however, is largely inaccurate and carries only historical meaning, as cleaving of DNA by photolyases is carried out by means of beta-elimination rather than by hydrolysis [69].

Repair by photoreactivation is typical of prokaryotes and single-celled organisms, as their exposition to UV irradiation is often unavoidable and there is no barrier protection available. In higher eukaryotes, the homologues of the prokaryotic proteins of repair by photoreactivation (cryptochromes, CRY) function as key components of the circadian molecular oscillator [31,194,195]. In lower eukaryotes, such as insects, cryptochromes may still function as photoreceptors, but mammalian CRY proteins have lost their direct photoreceptor function. In humans, there are two major homologues of photolyases, *CRY1* ((6-4)-photolyase) and *CRY2*, containing the flavine core and the pterin cofactor, but lacking photolyase activity [196].

Eukaryotic cells usually remove photoproducts from their DNA by excision of a fragment surrounding the lesion site and resynthesis of the DNA (NER). In some cases, when the UV-induced nucleotide modifications are recognisable by repair glycosylases, BER may be

employed[197,198]. For example, UV irradiation often produces pyrimidine hydrates which are readily recognised by endonuclease III (Nth) of E.coli.

3.1.2. Direct repair by T4 endonuclease V

The mechanism of direct DNA repair associated with T4 endonuclease V does not result directly in restoration of the initial DNA structure. Rather, it is a mechanism of activation of DNA repair by generating a signal for the repair machinery to assemble at the damage site. The substrate of T4 endonuclease V is thymine dimers in DNA. The enzyme actively scans the length of DNA for dimers by non-specific electrostatic interaction. Once the presence of a dimer is acknowledged, the 5'-pyrimidine is removed and the phosphodiester bond is cut at the resulting AP-site (Fig. 2). Thus, single-strand breaks are generated at the site of the initial damage, which present a powerful recruitment signal to the cellular DNA repair machinery. While thymine dimers may be left unrepaired in DNA for quite some time, breaks are usually promptly repaired. After the surrounding region, containing the remainder of the thymine dimer is excised, the DNA from the repaired strand is synthesised de novo and the free end is ligated. The size of the repair 'patch' is in the order of several nucleotides (in in vitro conditions, 4 nucleotides) [199].

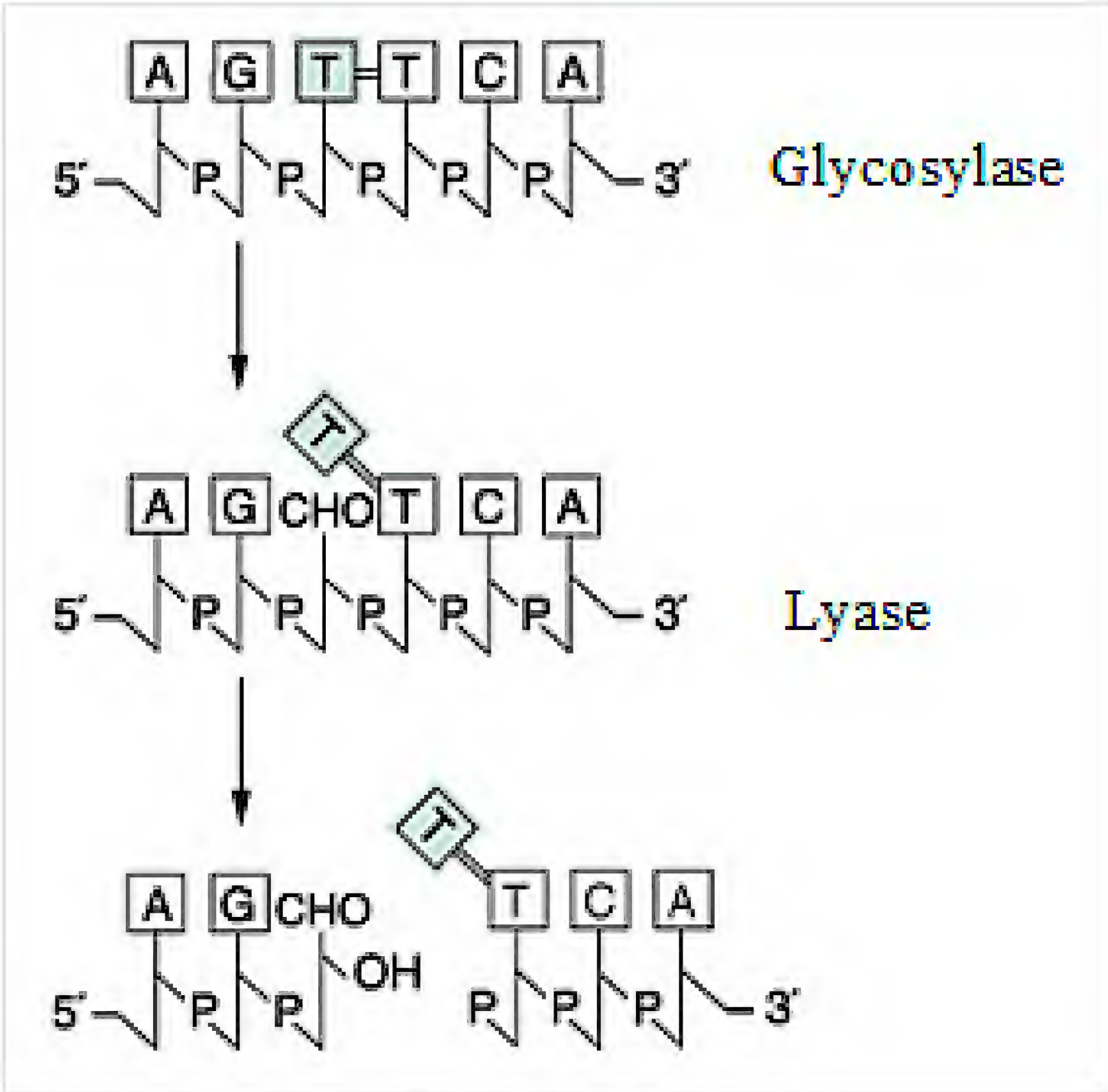

Figure 2. Mechanism of the catalytic action of T4 endonuclease V.

The capacity of T4endoV to transform and amplify the signal for presence of DNA damage has already found its applied use in post-irradiation sunscreening strategies [200, 201].

Unlike the conventional sunscreening approaches that work by prevention of excessive UV exposure (usually by creating an anti-UV barrier), post-irradiation strategies may be employed after the exposure has already happened. Certainly, post-irradiation treatments could not substitute completely barrier methods of sunscreening, but they may serve as a useful adjuvant to prevention of UV-related damage in healthy individuals, as well as in patients with diseases and conditions, associated with extreme UV-sensitivity.

3.1.3. Direct DNA repair by other mechanisms

Spore photoproduct photolyase

In spores after UV-irradiation are often generated specific, even unique photoproducts, such as 5-thyminyl-5,6-dihydrothymine, instead of cyclobutane dimers or 6-4 photoproducts [202]. Spore photoproduct photolyase uses the energy of visible light to break the C-C bond between two thymine residues and restore the initial thymines. The reaction requires the presence of S-adenosylmethionine as a cofactor [203].

O6-methylguanine DNA methyltransferase(O6-alkylguanine DNA alkyltransferase)

O6-methylguanine DNA methyltransferase works by transferring methyl groups from O6-methylguanine and other alkyl groups from various alkylated substrates to a designated cysteine residue in the active centre of the enzyme molecule [42,204]. The methylated protein is subsequently degraded via the ubiquitin-dependent mechanism. In prokaryotes, O6-methylguanine-DNA methyltransferase is capable of repairingphosphotriester adducts as well [205]. O6-methylguanine DNA methyltransferase is one of the major enzymatic activities of direct DNA repair in eukaryotic cells. The activity of the enzyme may be modulated in vitro and in vivo, increasing or decreasing the potential of some DNA damaging agents for causing damage (e.g. halogenated hydrocarbons, epihalohydrins, and others) [206,207]. The toxicity of some anticancer agents(for example, platinum derivatives) may be enhanced by modulation of the activity of O6-methylguanine DNA transferase [208,209]. Thus, the desired therapeutic effect may be achieved with lower doses of platinum compounds, provided that the target cells are sensitised to the anticancer compound by decreasing their capacity to repair the damage inflicted by the compound.

3.2. Mechanisms of excision repair

3.2.1. Base excision repair

Apurination is one of the most commonly occurring types of damage repaired by BER. Incidences of loss of purine bases happen on the average 10^3–10^4 times per day per human cell [210].Loss of pyrimidine bases (also repaired by BER) occurs with lower frequency. Uracil in DNA, resulting from hydrolytic deamination of cytosine is also repaired by BER. The BER pathway also processes alkylated bases and oxidised bases produced by ionising radiation or via the indirect action of reactive oxygen species.

Different types of damaged bases are recognised by specific glycosylases, which cleave the N-glycoside bond at the damaged base, creating an apurine/apyrimidine (AP)-site. The abasic site is then removed by an AP-endonuclease/AP-lyase, introducing a single break in DNA in 5'- or 3- direction, respectively, from the abasic site. The missing DNA is resynthesised, then the gap in the polynucleotide chain is ligated (Fig. 3).

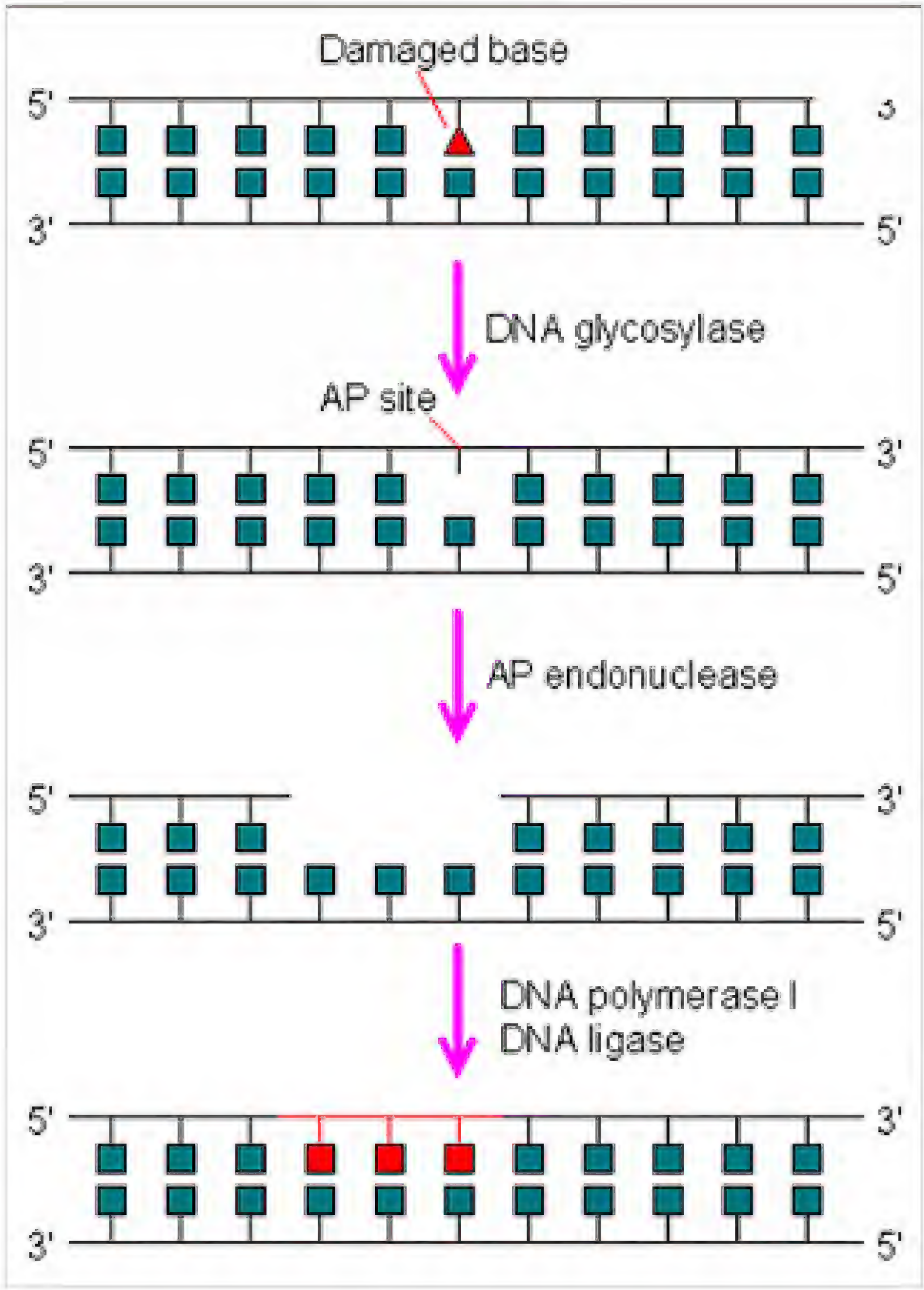

Figure 3. Essential steps in base excision repair.

Base excision repair for removal of oxidised or alkylated bases may be carried out in a 'short-patch' manner (replacing only the damaged nucleotide) or in a 'long-patch' manner, similarly to the mechanism of Okazaki fragment processing [11]. The initial phases of the long-patch mechanism are identical with the common mechanism. The action of a DNA N-glycosylase on the damage site produces an abasic site. It is then processed by an apurinic/apyrimidinic endonuclease that creates a nick in DNA. The resynthesis of DNA by the DNA polymerase displaces several downstream nucleotides, forming a 5'-flap, similarly to what occurs during the replication of the lagging strand. Then the flap is removed by FEN1 [30,211] and the nick is ligated by DNA ligase.

Until a decade ago, the common concept was that the gross defects in the genes coding for proteins acting in BER (e.g. loss-of-function mutations) are lethal in utero as no monogenic disease was known to be associated with defects in BER genes. Later, however, it was demonstrated in animal models that formamidopyrimidine glycosylase (Ogg1) plays a role in the expansion of the trinucleotide repeat implicated in the pathogenesis of Huntington's disease (HD) [212,213]. Ogg1-associated expansion occurs in somatic cells, as part of the normal phenotype of ageing. The relationship between carriership of certain allele variants of genes coding for products with roles in BER and the risk of development of various tumours was established around the same time [214]. Later, it was shown that some polymorphic variants of hOGG1 (the human homologue of Ogg1), MUTYH (the mammalian homologue of MutY, A/G-specific adenine DNA glycosylase) and G/T thymidine glycosylase (TDG)may modulate the transplant-related mortality in recipients of allogeneic haematopoietic stem cells [215,216]. It has been already proven, albeit in animal models only, that mutations in some of the human genes, coding for AP-lyases are associated with multifactorial disease, such as metabolic syndrome [217].

3.2.2. Nucleotide excision repair

After the damage in DNA is recognised (by different mechanisms, depending on the transcription status of the repaired region), NER begins with excision of a single-strand oligonucleotide fragment containing the damage site. The excision requires hydrolysis of two phosphodiester bonds, located 5'- and 3'- from the damage site, respectively. The excised nucleotide is released from the double helix and the resultant gap is filled by DNA synthesis, starting from the free 3'-OH end using the intact strand as a template. After the synthesis is complete, the single-strand break is ligated to the remainder of the repaired strand (Fig. 4).

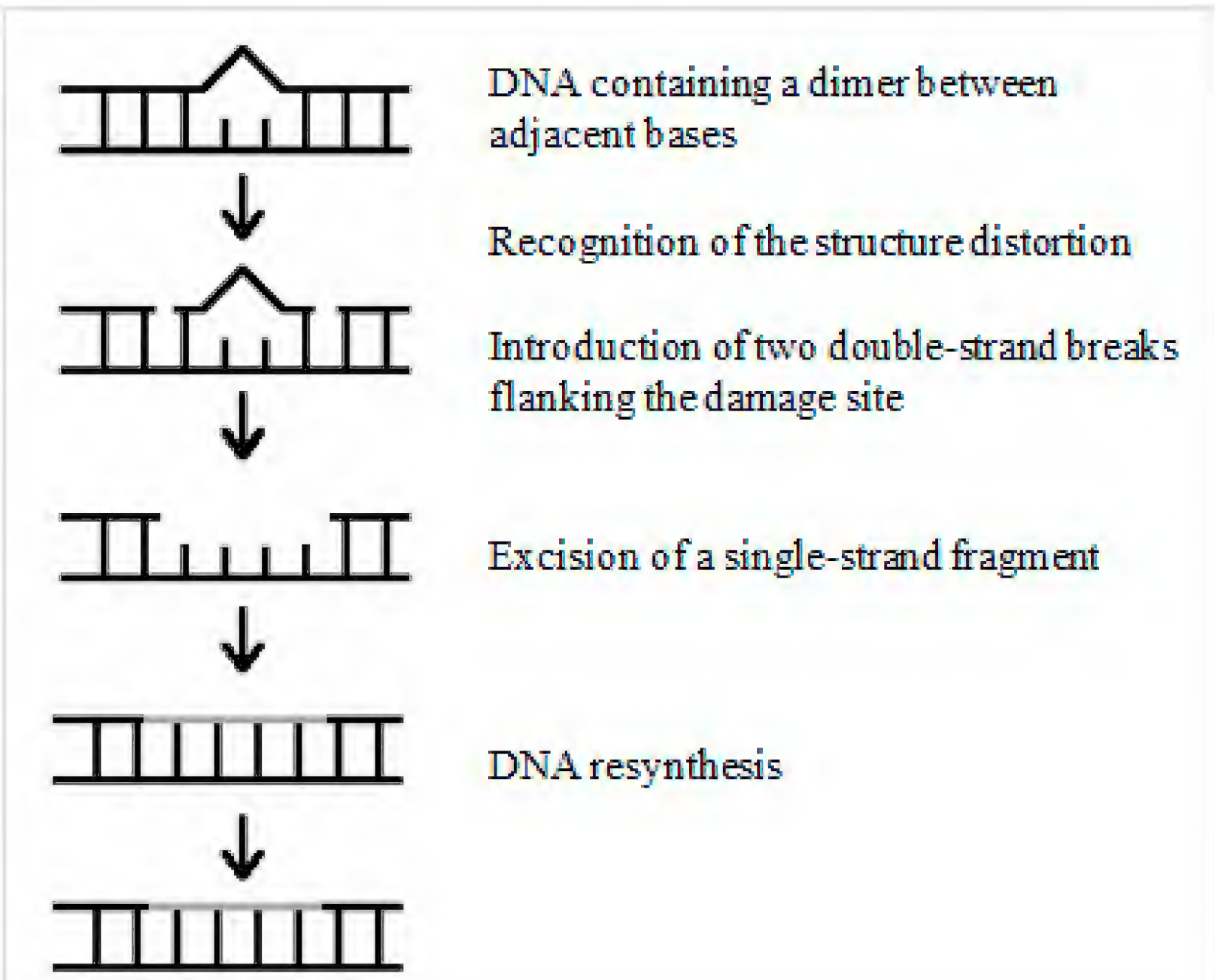
DNA containing a dimer between adjacent bases
Recognition of the structure distortion
Introduction of two double-strand breaks flanking the damage site
Excision of a single-strand fragment
DNA resynthesis

Figure 4. Essential steps in nucleotide excision repair.

The size of the excised fragments in NER is different in prokaryotes and in eukaryotes. Usually, it is the fifth phosphodiester bond that is cleaved in the 3'- direction from the damage site, albeit slight variance may exist (±3 nucleotides). In prokaryotes, the other incision is made at the eighth phosphodiester bond in 5'-direction from the damage site, while in eukaryotes it is the twenty-fourth phosphodiester bond that is hydrolysed in the 5'-direction (again, this may vary a little –from the 20^{th} to the 25^{th} phosphodiester bond) [218]. The length of the released oligonucleotides in prokaryotic NER is 12–13 nucleotides, while in eukaryotic NER the excised oligonucleotides are about 30 bp long [218–221]. To be more precise, in vitro, using cell-free extracts or purified excision repair factors, as well as in vivo, the length of the excised region in eukaryotes has been found to vary between 24–32 nucleotides[218].

NER is a very versatile repair mechanism, being capable of recognising and repairing a vast variety of types of DNA damage. Among the latter are, for example, cyclobutane pyrimidine dimers resulting from UV irradiation; benzopyrene diol epoxide-guanine adducts from tobacco smoke; psoralen-thymine adducts and guanine/adenine cisplatin derivatives resulting from chemotherapeutic treatments; mismatched bases, small (1–3 nucleotide long) loops in DNA; O6-methylguanine and other types of methylated bases; and even single-strand breaks [2,222]. NER may, at least partially, take over from BER and mismatch repair, as it is capable of repairing the same lesions. This may be the reason that BER and mismatch repair-associated diseases and conditions rarely become manifest until adulthood and the age of onset and phenotype may be modulated (to an extent) by environmental and other factors; while NER-associated diseases usually becomes manifest at an early age and follows a severe course. This very broad substrate specificity of NER, however, comes at a price, as there are specific cases (for example, base mismatch repair) in which the repair machinery may become confused as to where the damage actually is[2]. More specifically, NER could and would repair base mismatches, but there is no inherent mechanism to discriminate between the strand that carries the 'correct' sequence and the strand with the 'incorrect' nucleotide, it may actually excise the strand containing the 'correct' sequence and use the 'abnormal' sequence as a template for resynthesis of the missing fragment, thus making the altered sequence permanent [222]. Thanks to the existence of the designated mismatch repair mechanism that is capable of determining which strand is the 'original', NER may actually introduce a mutation in the process of repairing damage, but it occurs in very rare occasions.

Up to the end of the XX century it was believed that all proteins playing a role in NER proteins exist in a pre-assembled state in a large ready-to-use aggregate, termed "reparosome". Later, this model was disproved and the modern views are that the DNA repair apparatus is assembled from scratch at the damage site [3,223,224]. The assembly of the NER machinery at the damage site follows a sequential pattern, adding subunit after subunit in a strictly regulated manner [3,224,225]. The type of subunits and the order in which they are added to the complex is similar in prokaryotes and eukaryotes, but some crucial differences exist. These are reviewed briefly below.

3.2.2.1. General mechanism of prokaryotic NER

In E. coli, only three proteins are sufficient for repair by nucleotide excision –UvrA, UvrB and UvrC, together called the UvrABC complex [219,226]. UvrA and UvrB are DNA-binding proteins with affinity to damaged DNA. The former binds specifically to double-stranded DNA, while the latter is specific to single-stranded DNA. UvrA functions as a molecular matchmaker, bringing together two molecular species with affinity to each other (UvrB and damaged DNA) in an ATP-dependent reaction and stabilising the resulting intermediate [2,227]. Briefly, a heterotetramer made up of one homodimer of two UvrA subunits and one homodimer of UvrB subunits binds to the damaged site via a specific flexible beta-sheet hairpin motif rich in hydrophobic residues, in the UvrB subunits [228,229]. This induces conformational changes in the complex UvrA2:UvrB2: damaged DNA, facilitating the recruitment of UvrC [230]. The conformational changes are believed to be the time-limiting step of prokaryotic NER [231]. Then, UvrA dissociates from the UvrB2:DNA complex, allowing for binding of UvrC [2,232]. Upon binding of UvrC, one of the UvrB molecules leaves the complex and the 3'- break is introduced in the repaired strand [232]. This induces further conformational change in the DNA-protein complex, allowing UvrC to make the 5'-incision [233]. The excised oligomer is released from the double helix with the assistance of helicase II (sometimes called UvrD) and UvrC. Then, DNA polymerase I displaces UvrB and refills the gap using the intact strand as a template. The newly synthesised fragment is ligated to the original strand by DNA ligase (Fig. 5).

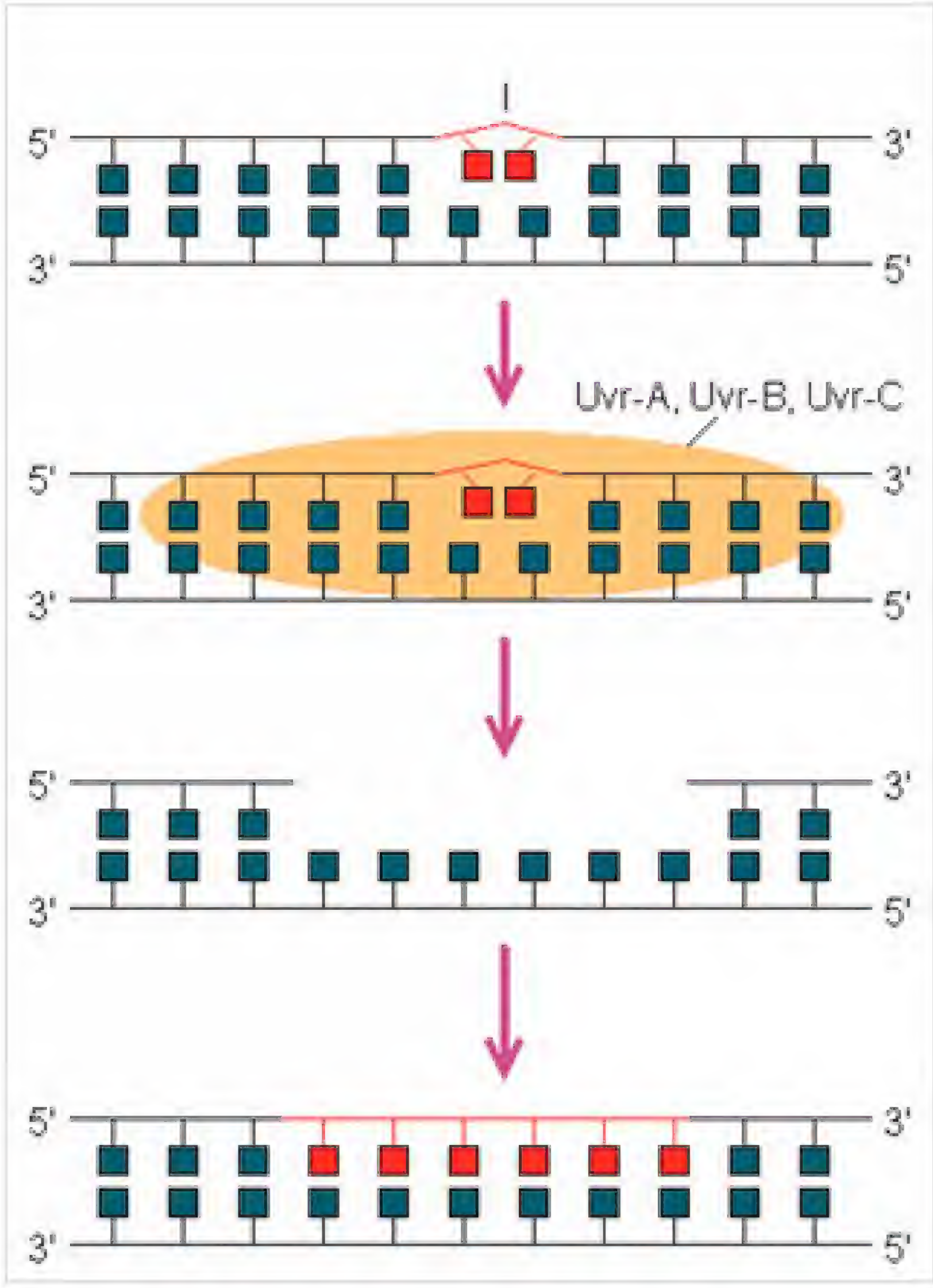

Figure 5. Basic scheme of prokaryotic nucleotide excision repair.

An alternative model for prokaryotic NER also exists, in which the damage is recognised in a two-step process [234–236]. The first step is the acknowledgement of the presence of a lesion-induced distortion of the DNA structure. Then the two strands are separated from each other in the vicinity of the distortion. In the second phase, the specific type of damage is recognised and repaired. The efficiency of repair in this model is dependent on the extent of bending and structure destabilisation that the lesion produces, with some lesions (producing more significant distortions) being repaired at a faster rate than others [236,237].

3.2.2.2. Eukaryotic NER – transcription-coupled repair vs. global genome repair

Eukaryotic repair by nucleotide excision requires the products of more than a dozen of different genes. Many of them share partial homology with the prokaryotic NER proteins, other are typical of eukaryotic cells only.

Repair by nucleotide excision has one distinguishing feature which makes it unique among all types of DNA repair. This is the correlation between the efficiency of repair and the transcription status of the repaired region. Namely, if the repaired DNA region is actively transcribed at the moment, the repair will be carried out with higher priority (at higher efficiency, that is, faster), while the non-transcribed regions are repaired with lower priority (lower efficiency, that is, slower).

Genetic mutations associated with deficiency in NER repair usually produce severe, early-onset disease phenotypes in man. Depending on the type of NER that is affected, the associated phenotypes may be different.

Eukaryotic genomes contain a huge amount of untranscribed DNA. In every given moment, only a relatively small fraction of DNA is undergoing transcription. Which regions exactly would be transcribed at a given time is dependent on the phase of the cell cycle and the stage of the life cycle the cell currently is. The transcribed genes are usually repaired with priority to the untranscribed DNA (transcription-coupled repair, TCR, TC-NER) [238–240]. The repair of DNA that is not actively transcribed at the moment is termed global genome repair (GGR).

It makes sense that transcribed genes are repaired with higher efficiency than the bulk of DNA, as any delay related to presence of damage in DNA is likely to affect adversely the functions of the cell. The transcribed strand may also be repaired more efficiently than the untranscribed strand of the same gene [239,241]. In different groups of organisms there may be differences in the ratio of TC-NER and GGR-related repair activities per cell. For example, GGR in rodents is usually carried out at a low rate, focusing the main repair efforts at the actively transcribed genes. This phenomenon is commonly known as 'the rodent repairadox' [22,239,242]. The genomic regions in which the chromatin is in 'open' conformation are also preferentially repaired to those in 'closed' conformation.

TC-NER and GGR are essentially the same mechanism, as they generally follow the same basic scheme. The only differences between them are in the properties of the target substrate (transcribed vs. untranscribed DNA) and the initial recognition of the presence of damage in DNA. The presence of RNA polymerase II stalled at the damage site is sufficient for initiation of TC-NER.Induction of GGR, however, requires the presence of signalling molecules at the damage site that would alert the repair machinery to the presence of damage. Usually, this function is carried out by the XPC-hHR23B complex, assisted by DNA damage-binding protein (DDB, part of a ubiquitin E3 ligase complex)[109,110]. XPC-hHR23B recognises the distortion of the double helix and relays the signal so that the assembly of the repair machinery is initiated at the damage site. A basic schematic of the differential mechanisms of initiation of TC-NER and GGR is presented at Fig. 6.

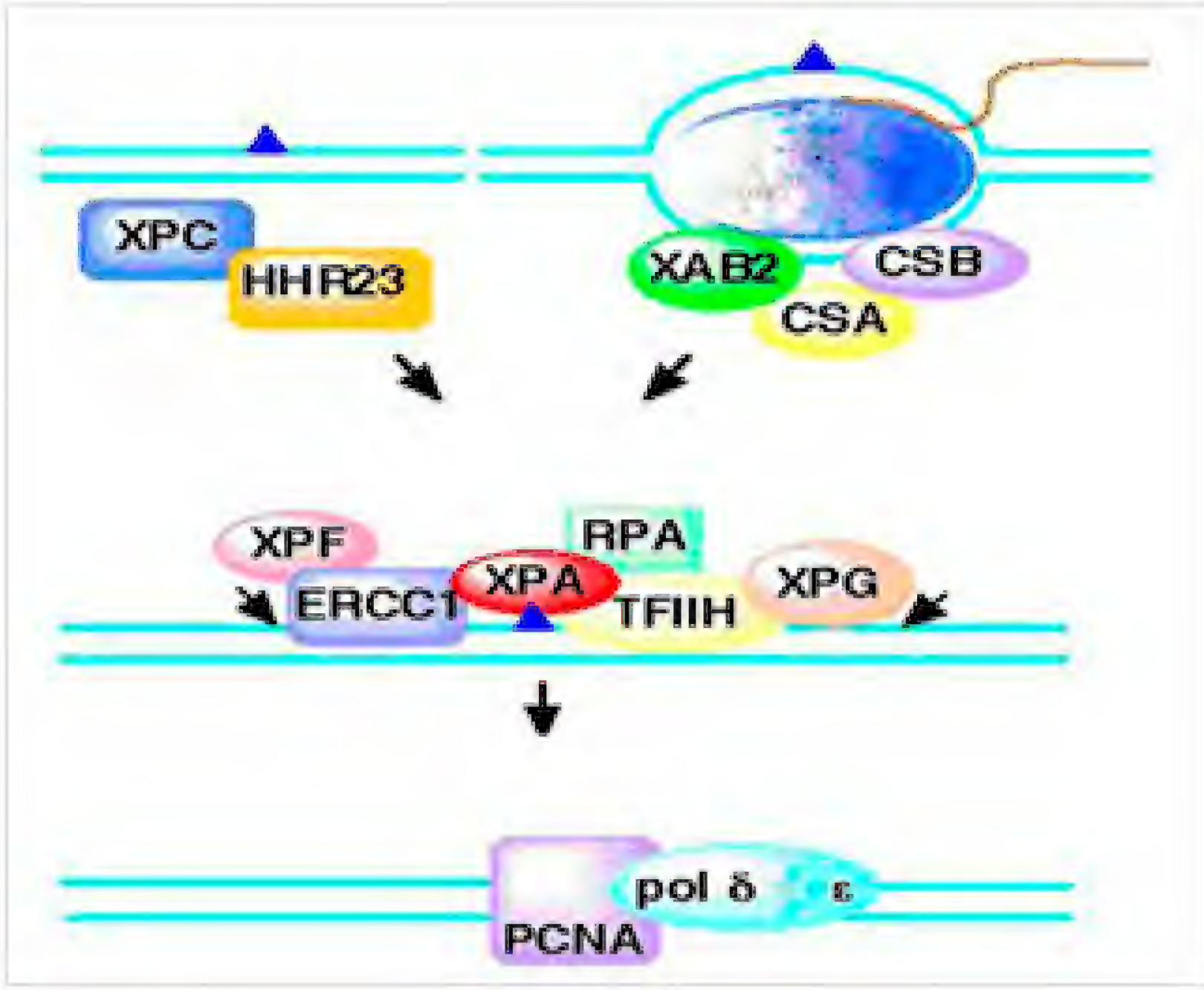

Figure 6. Differential initiation mechanisms of global genome repair (GGR) - left and transcription-associated repair (TC-NER) - right.

RNA polymerase II generally transcribes genes, coding for proteins. Therefore, in all active protein-coding genes, the presence of stalled RNA PolII at the site of damage would suffice as a signal for initiation of repair. Genes transcribed by RNA polymerases I and III (transcribing rRNA genes, tRNA genes and other genes coding for small RNAs) were initially considered not to be a subject of repair by TC-NER, but, rather, by the slower GGR mechanism [243–245]. Later, it was shown that DNA of ribosomal genes may be repaired by a transcription-coupled mechanism, depending on the packaging state of DNA in the rRNA-coding loci [246,247].

There may be differential pattern in DNA repair even between different transcribed genomic regions. This may be modulated by the type of damage. For example, the rate of repair of cyclobutane pyrimidine dimers in genes transcribed by polymerase I and II may be differential, with damage in protein-coding genes repaired at a faster rate than the damage in ribosomal genes. DNA adducts and interstrand crosslinks in genes, transcribed by RNA PolI or II, are, however, repaired with equal efficiency[245]. Even with the same general type of damage, the efficiency of DNA repair may vary. For example, the presence of thymine dimers (photodamage, produced by UV irradiation) usually triggers the activation of the TC-NER, while the repair of 6-4 photoproducts (also photodamage, produced by UV) is normally managed by the mechanisms of global repair [22].

A third type of NER, associated with the conformation of the chromatin domain in which the repaired region resides was described in 2002. It was termed differentiation-associated repair (DAR) [3,23]. Similarly to TC-NER, DAR is strongly associated with the transcription

state of the repaired region, but is not dependent on stalled RNA polymerase II to initiate repair, but, rather, on the conformation of the chromatin ('open' – the surrounding region is under transcription or 'closed' – the region is not transcribed at the moment). Using the DAR mechanism, the untranscribed (coding) strands of transcribed genes are repaired at a relatively high rate, regardless of the fact that RNA polymerase does not physically reach the untranscribed strands. DAR may be viewed as a mechanism for ensuring the integrity of the coding strand of the expressed genes.

3.2.2.3. Specificities of TC-NER in prokaryotes

In prokaryotes and eukaryotes alike, the sole presence of stalled RNA polymerase II at the damage site and/or the presence of abortive transcripts is believed to constitute a sufficient signal for recruitment of the NER repair machinery [248]. In prokaryotes, the stalled RNA PolII molecules are recognised by an ATPase protein called transcription-repair coupling factor (TRCF) [249]. TRCF is coded by the bacterial gene mfd (muta¬tion frequency decline) [250]. TCRF functions in bacterial TC-NER in recruiting the proteins of NER specifically to the transcribed DNA strand. It also binds to the stalled RNA polymerase II and causes its dissociation from the copied DNA strand. TCRF binds to the UvrA subunit of the UvrA2UvrB2complex and targets the complex to the damage site. Then, TRCF dissociates allowing UvrB to bind with high affinity to the damaged DNA. Further, NER follows the general scheme outlined in Fig. 6 [251].

The mammalian homologue of the gene *mfd* is *Csb*, or *CSB* in humans. Molecular defects in the *CSB* gene result in Cockayne syndrome complementation group B.

3.2.2.4. Specificities of TC-NER in eukaryotes

TC-NER is activated by the presence of stalled RNA PolII at the damage site. The acknowledgement of the presence of damage in DNA and the subsequent stalling of RNA polymerase II may be dependent on the propensity of the polymerase to misincorporate nucleotides into the transcript upon encounter with a DNA lesion. For example, upon entering of the 5'- thymine of a cyclobutane pyrimidine dimer into the active centre of the RNA polymerase II, the enzyme misincorporates uridine into the elongating transcript, blocking the translocation [252,253]. The stalled RNA polymerase II is subsequently tagged for degradation by the ubiquitin-dependent mechanism, clearing the site for the repair machinery [254,255]. RNA polymerase II is being stalled by a similar mechanism at cisplatin adducts, but the lesion is prevented from entering the active site and a non-template AMP is being misincorporated into the growing chain [256].

After the damage has been recognised, NER follows the general scheme on Fig. 6.

3.2.2.5. Specificities of GGR in prokaryotes

The mechanism of global genome repair in bacteria is based on the UvrABC system, described above.

3.2.2.6. Specificities of GGR in eukaryotes

The only difference between the TC-NER and GGR in terms of mechanism is, as we already saw, the initial damage recognition step. Global genome repair in eukaryotes consists of the following basic phases (Fig. 7):

- The distortion in the DNA structure at the site of the damage is recognised by the XPC–hHR23B complex;
- The transcription factor TFIIH is recruited at the damage site. The helicases XPB and XPD, responsible for the unwinding of the double helical structure, are subunits of TFIIH;
- The complex XPA-RPA is loaded, which, in turn, allows the structure-specific nucleases ERCC1-XPF and XPG to introduce the 5'- and the 3'- incisions, respectively;
- DNA polymerase δ or ε and accessory proteins (replication factor C (RF-C and the proliferating cell nuclear antigen (PCNA) work together to fill the gap, using the intact strand as a template [117];
- The 3'-end of the newly synthesised DNA fragment is ligated to the 5'-end of the 'old' strand by DNA ligase III or ligase I.

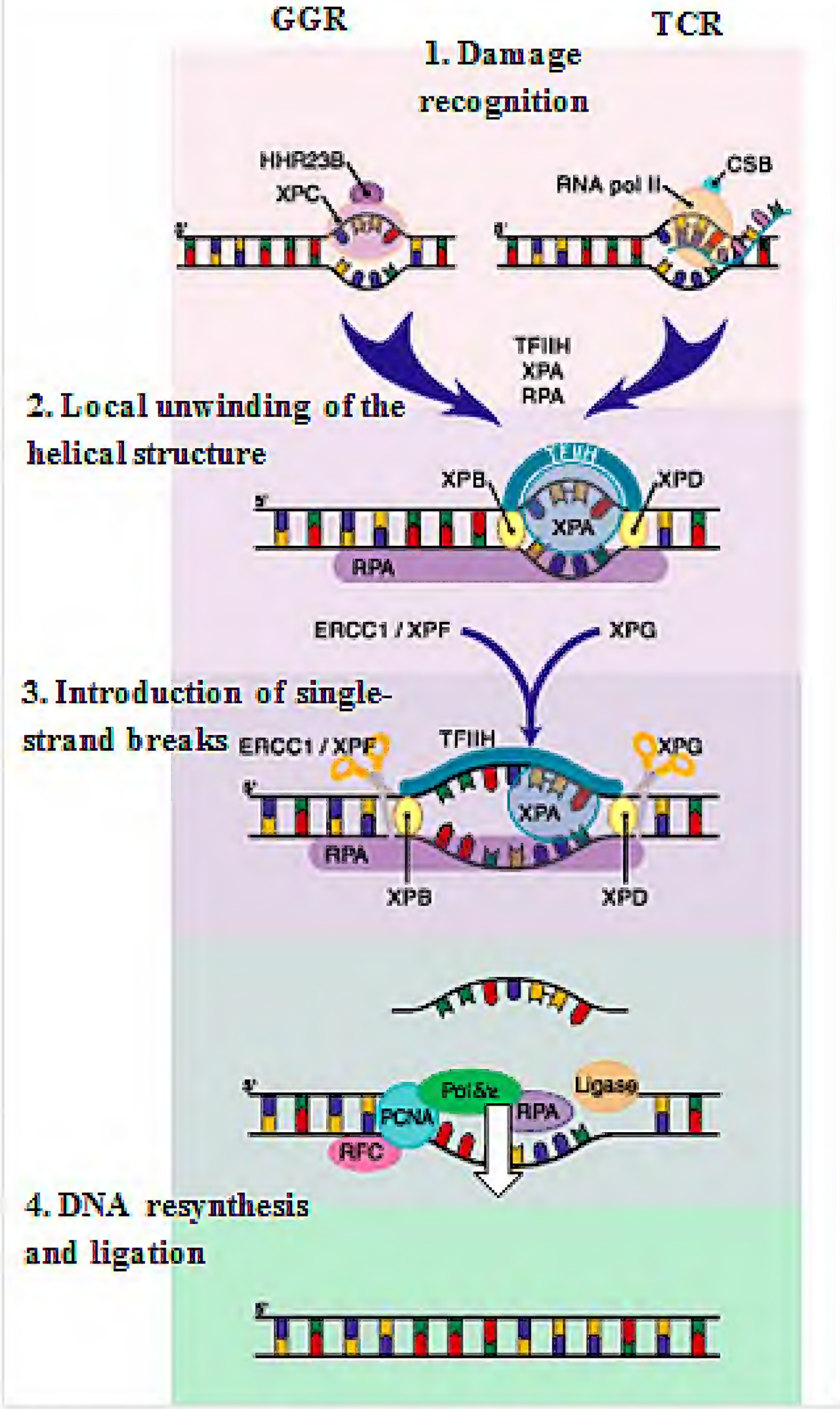

Figure 7. Stages of eukaryotic NER.

Initially, it was believed that XPA was the earliest NER protein to recognise and bind damaged DNA. Later it became clear that XPA, complexed with RPA (repli¬cation protein A)

joined the assembling NER complex rather late, after TFIIH has already been recruited [224]. XPC–hHR23B is the complex that comes first in contact with the damage site and generates the primary activation signal for the NER machinery of the cell. It has very high affinity to single-strand DNA and is capable of autonomous recruiting the remaining components of the pre-incision complex, including TFIIH [110,224,257]. XPC-HR23Bprovides a common link between the mechanisms of BER and NER. It has been found to interact physically as well as functionally with thymine DNA glycosylase (TDG) and stimulating its activity [258,259]. XPC also plays a role in the p53-dependent process of decision-making about whether to attempt repair of DNA damage or to reroute the cell directly to the apoptosis pathway(by regulating the expression of some of the anti-apoptotic caspases) [260,261].The expression of XPC is directly regulated by wild typep53 via designated response element in the XPC promoter [262,263].

The actual act of recognition of DNA damage site is implemented by RPA component of the XPA-RPA complex [234,264]. RPA has a DNA binding site on single-stranded DNA which spreads over 20–30 nucleotides, that is, it covers the repair site almost completely [265]. XPA binds the damaged site in the presence of RPA. The complex XPA-RPA binds to the XPF-ERCC1 heterodimer and stimulates the endonuclease activity of XPF and XPG [224, 266].

The helicases XPB and XPD enter NER as subunits of the basal transcription factor TFIIH. TFIIH consists of ten subunits, seven of which make up the core (XPB, XPD, p34, p44, p52, p62 and TTDA (GTF2H5)) and three comprise the kinase domain (CDK-activating kinase, CAK) [108,267,268]. The functionality of the helicase subunits of TFIIH is crucial for prevention of abortive termination of nascent (newly synthesised, 15–20 bp long) mRNA [269,270]. After TFIIH had arrived at the site of the damage, the nucleases XPF-ERCC1 complex and XPG make their two single-strand incisions, XPF cutting in the 5'- and XPG in the 3'- direction from the damage site. The XPF/XPG duo then acts to remove the Y-shaped structure formed by the partially single-stranded region of the DNA molecule, resulting from the action of the repair machinery [111,271].

Polymerase activities in eukaryotic NER are provided by DNA polymerase δ and ε(unlike BER, where the major polymerase function is carried out by DNA polymerase β) [89,114,115,117]. Polymerase β is actually fully capable of carrying out DNA synthesis of a previously excised fragment, but as soon as PCNA binds to the free 3'-OH end of the repaired strand, it becomes inaccessible to DNA polymerase β [272].

After the missing DNA fragment has been resynthesised, its 3'-OH end is ligated to the 5'-phosphate of the adjacent nucleotide by DNA ligase III, complexed with its stabilising factor XRCC1 or by DNA ligase I (in dividing cells) [102,122,273].

Mechanisms of mismatch repair

Mismatch repair is, in fact, repair by excision. Unlike NER, however, in which any of the two strands (either the damaged or the undamaged strand) may be subject to excision, mismatch repair specifically targets the strand carrying the altered (mismatched) nucleotide (similarly to BER). The targeting mechanism may be principally different in prokaryotes and in eukaryotes (see below).

3.2.3. Nobody's perfect, or why do we need a designated mechanism for mismatch repair

The need for a specific mechanism to recognise and repair mismatched nucleotides stems from the inherent error-proneness of DNA polymerases. Even the replicative polymerases may add the wrong nucleotide sometimes (albeit rarely). Usually, they excise it right away, repeat the joining step, and if they get it correct this time, the synthesis continues as usual. If the second added nucleotide is not a match again, it is excised and the attempts to add a matching nucleotide are repeated until a match is found. Sometimes (very rarely) the mismatched nucleotide is not excised, but the synthesis goes on, producing a daughter strand carrying a mismatch.

The DNA polymerase cannot actively choose the type of nucleotide it ought to add next, rather, it, picks up any of the available nucleotides at random and catalyses its joining to the growing chain, regardless of the pairing properties of the opposite nucleotide. In case there is no match, the polymerase hydrolyses the newly created phosphodiester bond using its 3'-5'–exonuclease (proofreading) activity, releasing a nucleoside monophosphate and regenerating the 3'-OH free reactive end of the chain, so that the cycle may start over.

There are exceptions to the random principle of picking nucleotides to add next. For example, some DNA polymerases will preferentially add dAMP opposite abasic sites in vitro as well as vivo (known as 'the A-rule') [274,275].

It is fairly obvious, however, that the DNA polymerase is bound to add the 'wrong' nucleotide approximately 3 times more often than the 'right' nucleotide (if we assume that all nucleotides are available and that they are in roughly equal amounts). The rate with which the proofreading activity of a DNA polymerase misses a mismatched nucleotide is constant for any given type of polymerase. This rate is often termed fidelity (copying fidelity) of the DNA polymerase. DNA polymerases are often subdivided into classes according to their fidelity.

The fidelity of polymerases is generally expressed as the mutation rate per base pair per doubling cycle. The in vivo fidelity of prokaryotic polymerases of replication varies between 10^{-7} and 10^{-8}. The error rate of eukaryotic DNA polymerases of replication are believed to be at least that accurate and possibly more accurate [276–278].

Fidelity is a very important parameter in in vitro DNA synthesis, with low-fidelity polymerases as Taq polymerase making copying errors roughly at 1:45,000 nucleotides (about $2.2x10^{-5}$) per doubling cycle, while high-fidelity polymerases such as Pfu and engineered high-fidelity Taq add a mismatched nucleotide at a rate about 2.5:1,000,000nucleotides($2.5:10^{-6}$) per doubling cycle. This means that in a fragment of 1000 bp, the low-fidelity polymerase will make at least one mismatch error in every 40–50 synthesised full-length fragments per doubling cycle. Experiments show, however, that the error rate may vary greatly between different amplicons–from ≈1:5000 to >1:80,000 per nucleotide per cycle [279]. In most PCR applications this would not matter significantly, unless, of course, a critically important mismatch (e.g. disrupting an important restriction site or introducing a new one) occurs in the early amplification cycles and is copied then again and again, until the majority of the resulting molecules contain the error.

In order to better illustrate the importance of mismatch repair in the context of error-proneness of replicative DNA synthesis, let us review a simplified situation in which a short fragment of DNA is copied and see whether the accuracy of copying is satisfactorily

high[280,281]. We would allow for random distribution of mismatches (that is, the chances for introduction of mismatches are the same for all nucleotides and along the length of the synthesised molecule), which never actually happens in vivo. Thus, we could calculate the ratio of the newly synthesised molecules which have sequence identical to the template molecule. Certainly, these calculations are purely hypothetical and would work for short fragments only.

If the efficiency of the synthesis is ƒ, the fraction of the template DNA (T), and the fraction of the newly synthesised DNA (N) may be expressed as:

$T = 1 / (f+1)$,

$N = f/ (f+1)$,

where ƒ varies between 0 (no DNA synthesis at all) and 1 (all synthesis on all templates is proceeding at 100% efficiency). The latter never actually happens, as at least some of the templates may be damaged or uncopiable(for any reason), agents inhibiting the polymerase activity may be present, etc.

The probability for synthesis of new molecules which are 100% identical with the template (p) may be expressed as:

$p = e^{-mL}$,

where m is the mutation (mismatch) rate and L is the length of the amplicon (in nucleotides).

All mismatches occur in the newly synthesised strands. Therefore, at the end of the doubling cycle, the fraction of the DNA strands with sequence identical to the template strand i would be:

$i = T + pN = (1 + f\, e^{-mL})/ f + 1.$

After n DNA doublings, the fraction of the DNA fragments with sequence identical to the template sequence I(n) would be:

$I(n) = i^n = (1 + f\, e^{-mL})^n/ (f + 1)^n.$

Let us take a short DNA fragment, about 300 bp long. At $f = 0.75$ and the mutation rate being close to that of the Taq DNA polymerase, about 2×10^{-5}/nucleotide/doubling cycle, every cycle would generate about 0.25% molecules which are not identical to the template. After 20 cycles, about 5% of the synthesised fragments would carry sequence which is not identical at least in one position with the template molecule. At 30 cycles, which is the usual number of cycles in a PCR experiment, nearly 8% of the newly synthesised molecules would have sequence non-identical to the initial template sequence.

Surely, in vivo the mutation rate is significantly lower and the value of ƒ is close to 1 (which is actually made possible by the presence of repair mechanisms). The fragments synthesised in replication are significantly longer, at least in the leading strand (in the order of 10^5bp). Therefore, if for the sake of simplicity we consider a 10^5 bp-sized fragment, error rate of polymerases about 5×10^{-9}(using the mutation rate per base per generation), f=0.99 (which is actually high) and use the same formula as above, the proportion of molecules which are identical to the template after one cycle of replication would be 99.975%. The number of mitotic cycles that a normal human somatic cell is capable of before reaching the Hayflick's limit is about 50, which makes for 98.75% of DNA fragments in the end being identical to the initial template molecule, and about 1.25% with at least one deviation from

the original blueprint. In a genome of $2.5x10^9$ bp (roughly the size of the haploid human genome), this makes up for over 30Mb of altered DNA. This accounts for mismatches in DNA only, and not other types of damage, some of which occur at very high rate every day. The size of the largest human chromosome (chromosome 1) is about 250 Mb, and the smallest chromosome (chromosome 21) chromosome – 47 Mb. Now the huge amount of 'junk' DNA in eukaryotic genomes begins to make sense, as the higher the amount of non-coding, 'disposable' DNA, the lower the risk for any damage event to occur within important coding sequence. Of course, this is all purely theoretical, but it gives an idea about the importance of DNA repair in the life of the cell, and especially of mismatch repair. Obviously, unless a watchdog system for replication-associated faults operates in every living cell, every cell division would introduce new mutations in DNA and eventually the DNA of living cells would hardly resemble the initial blueprint after several dozen divisions (and that even without the effect of other types of DNA damage). As it is, the chance for introduction of mutation/s per cell doubling is very low indeed, about one or two events per cycle of replication in a 10^9 bp–sized genome. These rare events, however, accumulate with increased number of cell doublings–that is, as age advances or, in special cases when other events occur (e.g. increased cell turnover), it may happen earlier.

Not all DNA polymerases in living cells have same or even similar fidelity. Depending on their functions, different polymerases may have very different error rates. As a rule, a typical replicative DNA polymerase would not copy a damaged template; rather, it would stall at the damage site and signal for recruitment of the repair machinery (though there are rare exceptions to this). Some special DNA polymerases, among which are the already mentioned polymerases eta, zeta and iota may allow for read-through replication of damaged templates with higher risk for introduction of errors than the replicative polymerases [181,282] (for more information, see below).

3.2.4. Mismatch repair in prokaryotes

For recognition and repair of DNA mismatches prokaryotes need only four repair proteins – MutS (recognises the mismatch), MutL (recruits and assists in binding of the endonuclease MutH to the site of the mismatch), MutH (endonuclease), and MutU (helicase II) [119,283]. E. coli strains carrying defects in these genes exhibit very high spontaneous mutation rate.

The repair of mismatches targets the newly synthesised DNA strand and is routinely activated after the replication is complete, as a final proofreading mechanism. The mechanism of tracking specifically the changes in the newly synthesised DNA strand is based on the presence of N6-methylated adenine in specific short sequences– d(GATC) [284]. As this modification is carried out after the replication of DNA is complete, it is believed that the temporary existence of one of the two strands in a DNA molecule in non-methylated state is the signal for the mismatch repair machinery to lock specifically onto it. Thus, the risk for introduction of mutations in DNA is minimised, as the sequence of the newly synthesised strand is corrected using the methylated mother strand.

A schematic of prokaryotic MMR may be viewed in Fig. 8. There are several variants of MMR, depending on the size of the excised region. The figure below presents the 'classic' long-patch repair by MMR.

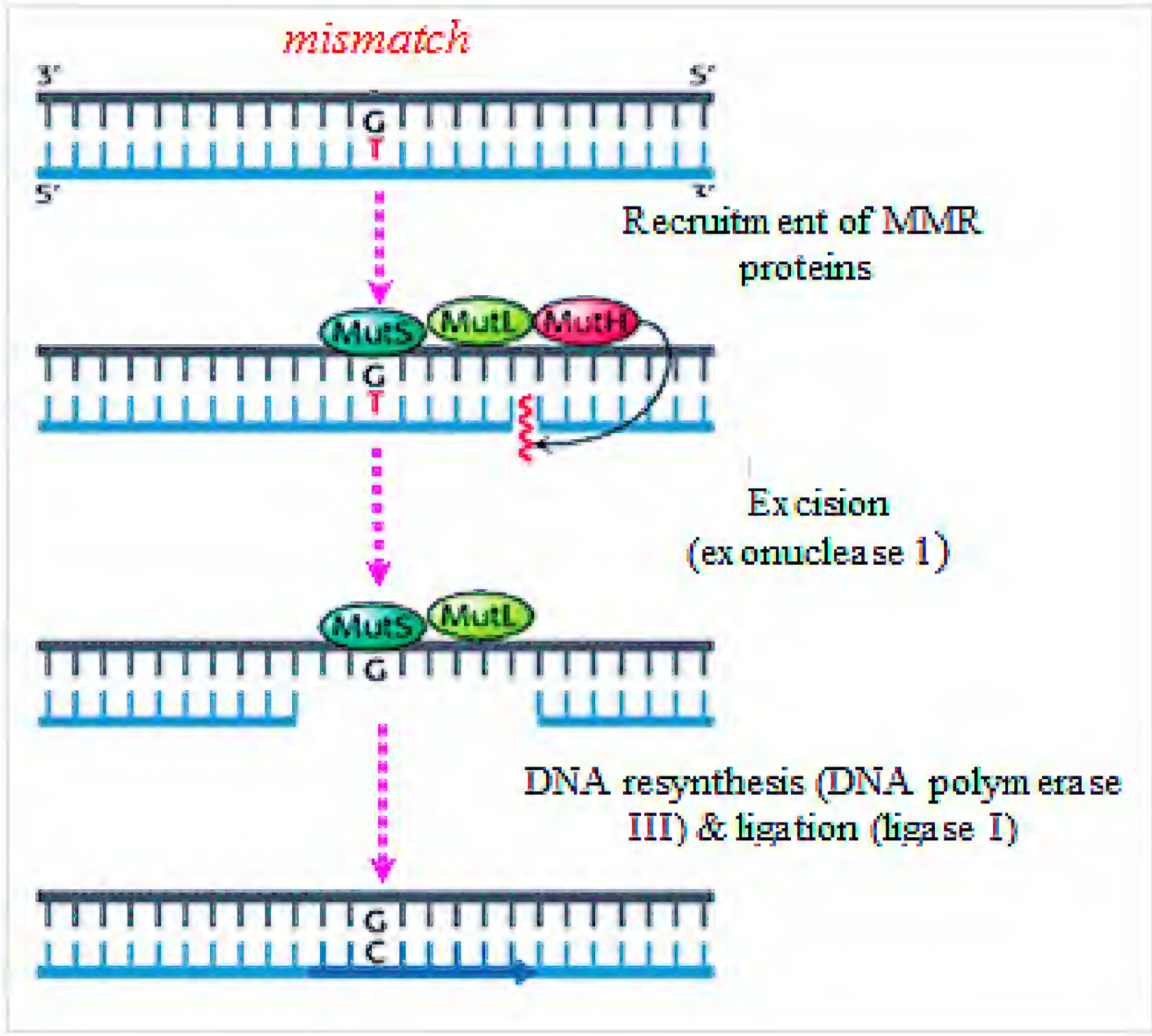

Figure 8. Phases of long-patch mismatch repair in bacteria.

Mismatch repair in prokaryotic cells occurs in several distinct phases [285,286]:

- The mismatch is recognised by a MutS homodimer;
- MutL joins in, signalling for recruitment of the MutH at the mismatch site;
- The endonuclease activity of MutH is activated, introducing single-strand break in the strand carrying the mismatch, in the closest to the mismatch hemimethylated d(GATC) sequence;
- The region containing the mismatch is excised in an ATP-dependent reaction involving MutS, MutL, the helicase MutU and a specific exonuclease (exonuclease 1), which completes the excision of the fragment containing the mismatch regardless of whether the previously recognised and cut d(GATC) sequence is located in 5'- or 3'-direction to the mismatch.
- The excised region is filled by DNA polymerase III, using the methylated strand as a template;
- The free 3'-OH end of the newly synthesised DNA fragment is ligated to the 5'-phosphate of the adjacent nucleotide by DNA ligase.

The single strands are kept separate during repair by single-strand binding proteins (SSBPs). Apparently, mismatch repair strongly resembles NER, the basic difference being the specification of the newly synthesised strand as substrate for repair. Also, since the site of the incision depends on the location of the nearest to the mismatch hemimethylated

d(GATC) sequence, the size of the excised fragment may vary, calling for mismatch repair systems with different 'patch' lengths (similarly to BER). The 'classic' long patch mismatch repair described above may replace up to a 1 Kb of DNA and requires all of the listed proteins–MutH, MutL and MutS as well as MutU, as repair of such a long region would require significant unwinding of the double helix. At least two accessory mismatch repair pathways are currently known to exist – namely, 'short patch' and 'very short patch' MMR. The 'very short patch' MMR has so far been observed in prokaryotes only. The other, 'short patch' may sometimes be employed by eukaryotic cells. Unlike 'long patch' mismatch repair mechanisms, the other two are more similar to base excision repair (BER) than to NER [285].'Short patch' mismatch repair removes A from mispaired A:G, A:C, C;C A:8-oxo-G and T from G:T mispairs. It makes use of a BER protein, MutY (bacterial A/G-specific adenine glycosylase, the prokaryotic homologue of eukaryotic G/T thymidine glycosylase) [287,288]. In bacteria, after the presence of adenine mispairs in bacterial DNA has been recognised, and in eukaryotes, in the presence of C:C; G:T and A:8-oxo-G mispairs, MutY (or its mammalian homologue MUTYH, respectively) deploys its glycosylase activity to remove the mispaired base from DNA. Usually, this is coupled with an apurinic/apyrimidinic endonuclease/lyase activity (endonuclease VIII (Nei) in prokaryotes, Ape1 in mammals). The endonuclease/lyase introduces a break in the strand carrying the abasic site, immediately adjacent to the 5'-side of the mispair, and utilises its exonuclease activity to excise a fragment containing the damage [84]. Short patch repair pathway results in excision of a fragment≤10 nucleotides in length [289,290].

The very short patch repair (VSP) MMR is a prokaryotic mechanism operating directly on mispaired nucleotides without excision of the surrounding region. It restores the original G:C pair in G:T mismatches in bacteria, usually occurring because of deamination of 5-methylcytosine [291].

3.2.5. Mismatch repair in eukaryotes

The mechanism of mismatch repair in eukaryotes is generally similar to the prokaryotic mechanism. The proteins involved are homologues of the respective basic prokaryotic proteins.

Several human homologues of bacterial MMR proteins have been identified. Human MutS and MutL homologues works as heterodimers made of two similar proteins, unlike the bacterial homologues, which are usually homodimers [292,293]. MutS has several eukaryotic homologues, named, respectively, MSH1 – MSH6. Human MutS heterodimers playing a role in DNA mismatch recognition are hMutSα (MSH2-MSH6) and hMutSβ (MSH2-MSH3), respectively. hMutSα usually recognises single-base mismatches while hMutSβ preferentially recognises larger mispaired regions [294]. The process is ATP-dependent.

Several human MutL homologues (hMLH1,hMLH3, hPMS1, and hPMS2) have been identified, which form several different types of heterodimers (hMutLα (MLH1:PMS2), hMutLβ (MLH1:PMS1), or hMutLγ (hMLH1:hMLH3), respectively) [295–297].

Specific human MutH homologue has not been identified yet but the human protein which resembles partially MutH and MutY–MUTYH functions as a A/G-specific adenine DNA glycosylase, excising oxidised and mispaired bases– e.g. 2-hydroxyadenine and adenine from A:G and A:oxo-G from DNA [298,299].

Human mismatch repair may be reconstituted in vitro using only the following set of proteins: MutSα(MSH2:MSH6) or β (MSH2:MSH3); MutLα (MLH1:PMS2); the single-strand binding protein RPA; exonuclease-1 (Exo1);replication factor C (RF-C); DNA polymerase delta; PCNA and DNA ligase I. The non-histone protein HMGB1 functions in mismatch repair, assisting in the recognition of the damage, as it interacts physically with MutSα and is required at a step prior to the excision of mispaired nucleotide [300].

The basic difference between prokaryotic and eukaryotic MMR is believed to be in the signalling mechanisms for the presence and the recognition of the mispaired base. The exact triggers for activating eukaryotic mismatch repair are still not entirely clear, as the methylation patterns in eukaryotic DNA follow very different rules from those in bacterial DNA; the length of DNA that must be scanned is huge; and the strand, bearing the mismatch, must be targeted specifically [125,294,301–303]. It has been proposed that in yeast the homologue of MutS – MutSα actively searches for DNA lesions, while the MutLα (the homologue of bacterial MutL) searches for lesion-bound MutSα [304].

Inherited defects in mismatch repair pathways promote genomic instability by increasing the rate of spontaneous mutation in the genome. Mutations in the some of the human homologues of MutS and MutL (*MLH1*, *MSH2*, *MSH6*) result in a cancer-prone phenotype. No human disease has been identified to be associated with defects in the *EXO1* gene yet, but it has been reported that inactivation of the *Exo1* in knockout mice results in DNA mismatch repair defects, sterility due to meiotic failure and, again, increased susceptibility to cancer [305]. Three polymorphic variants of human EXO1 (E589K, E670G and H354R) have been identified to be associated with poorer prognosis (lower survival) in patients with lung cancer, treated with platinum derivatives [306].

3.3. Mechanisms of repair of double-strand DNA breaks

... what's always the last thing to mend,
the middle of middle and end of the end?
J. K. Rowling. Harry Potter and the Goblet of Fire (2000).

Double-strand breaks (DSBs) may occur in DNA under the influence of exogenous factors (ionising radiation, some chemotherapeutics (e.g. radiomimetics), etc. In tumour cells, generation of excess DSBs may be result of genomic instability. Oxidative stress (via reactive oxygen species) is also a major source of double-strand breaks. Since persistence of double-strand breaks in the cell's DNA may have very serious consequences, the tolerance of healthy cells for DSBs is usually very low, the typical limit for activating the apoptosis programme being 3–4 DSBs per genome. In physiological settings, introduction of double-strand breaks may be normal part of the life cycle of some types of cells (for example, somatic recombination of immunoglobulin genes and TCR receptor genes; meiotic recombination; switching of mating types in yeast, movement of mobile genetic elements around the genome, etc. Endogenous introduction of double-strand breaks is usually under strict control, with the apoptosis mechanisms primed and alert in order to eliminate any cell that has sustained damage above certain threshold.

A double-strand break in DNA can principally be repaired via two different mechanisms – recombination with homologous DNA (homologous recombination, HR) or by joining together non-homologous sequences (non-homologous end joining, NHEJ) [307]. Both

mechanisms are strictly controlled at multiple points so as to prevent accidental and/or untimed activation of related repair pathways; accumulation of potentially toxic intermediates; replication blockade; unscheduled or erroneous recombination and/or chromosome fusion and breakage.

Repair by homologous recombination is usually employed by cells in the synthetic and postsynthetic (S and G2) phases of the cell cycle, where sister chromatids may be used as templates [308,309]. Non-homologous end joining is usually used for repair purposes in non-dividing cells, as well as in cells which are capable of division but are past the S and G2phases of the cell cycle.

The fidelity of DNA synthesis associated with repair of double-strand breaks is lower that the fidelity of replicative DNA synthesis. Deem et al. estimate the fidelity of DNA synthesis in DSB repair to be about 10^3–10^4 lower than the fidelity of DNA replication [310].

3.3.1. Repair by homologous recombination(HR)

The general mechanism of homologous recombination may be divided into several sub-pathways – double-strand break repair (DSBR), synthesis-dependent strand annealing (SDSA), and break-induced replication (BIR or break-copy replication) [311]. Generally, all three mechanisms require the presence of a homologous template DNA molecule and a single – stranded 3'-OH end to initiate the invasion and pair to the homologous template.

Each of the mechanisms may result in gene conversion, that is, substitution of one DNA sequence for another and loss of heterozygocity.

Double-strand break repair (DSBR)

When there are areas of homology between the sequences containing broken DNA ends, more than one mechanism may be engaged to ensure that repair is carried out without loss of genetic information or that the sequence of the region in the vicinity of the DSB is recovered with minimal losses or alterations. Repair of double-strand breaks by homologous recombination may be broadly subdivided into three phases: presynthetic, synaptic and postsynthetic (Fig. 9) [312,313]. There are variations of the mechanism (see below), but the working principle is essentially the same between prokaryotic and eukaryotic cells.

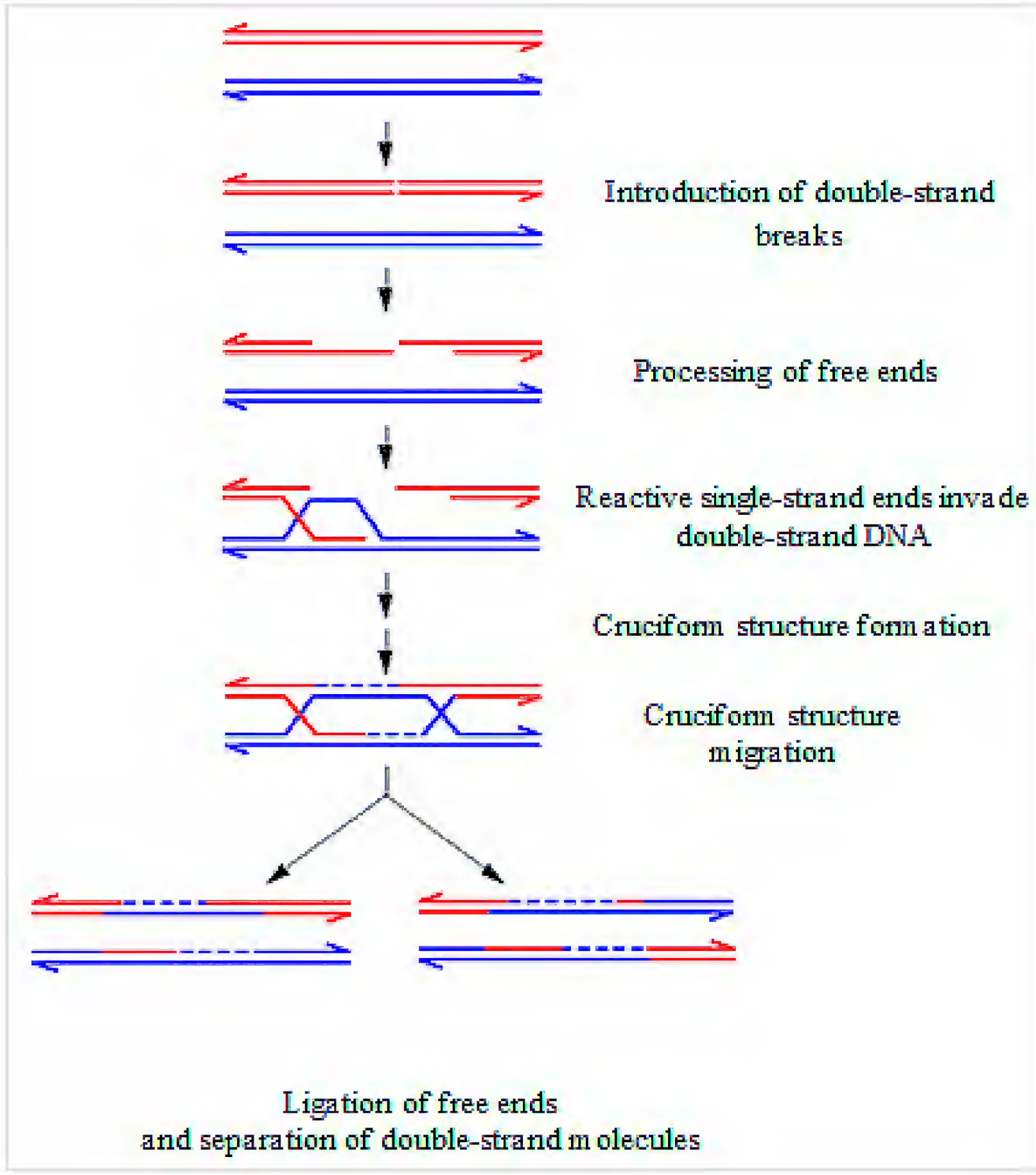
Introduction of double-strand breaks
Processing of free ends
Reactive single-strand ends invade double-strand DNA
Cruciform structure formation
Cruciform structure migration
Ligation of free ends
and separation of double-strand molecules

Figure 9. General scheme of repair of double-strand breaks by homologous recombination.

- After the introduction of a double-strand break in one of the recombining molecules, the presynthetic phase begins with processing of the resulting ends in order to generate single-strand tails, carrying the reactive free 3'-OH groups (end resection). This requires the presence of exonucleases (in eukaryotes, Mre11) and helicases, as the DNA regions involved in recombination must be unwound. In case the DSB is introduced secondary to the damage that must be repaired, or in cases of physiological homologous recombination (e.g. V(D)J recombination), endonuclease activities may be employed to introduce the double-strand break in DNA.
- The synaptic phase includes recognition of homologous DNA sequence, the invasion of a single-stranded tail into the homologous duplex and the initiation of DNA synthesis from the 3'-OH ends using the non-broken DNA molecule as template. This phase requires specific exonucleases and DNA polymerase activities, as well as single-strand binding proteins [reviewed in 314].

- The postsynthetic phase includes the formation of a cruciform structure (also called Holliday structure); the migration of the cruciform structure along the length of DNA, extending the length of the recombining region; the resolution of the cruciform structure and, finally, the separation of the two DNA molecules.

  The resolution of the cruciform structure requires specific enzymatic activities termed as resolvases (recombinases). Among these, for example, is the already mentioned bacterial protein RuvC, but there are other types of recombinases as well.
- Finally, free ends are ligated by ligase. In eukaryotes, ligase I or ligase III may be employed [161,315].

DSBR may generate non-crossover (the template strand exits the recombination unchanged) or crossover products between the template DNA molecule and the molecule undergoing repair. In DSBR, there may be substitution of all the DNA sequence beyond the break site.

Single-strand annealing (SSA)

This is another mechanism for repair of DSBs that requires a homologous DNA sequence as a template, but not necessarily on a separate DNA molecule [316,317]. SSA uses repeated sequences within the same DNA molecule as sources of partial homology. It is error-prone, as is NHEJ, and bears significant mutagenic potential, not only because the DNA polymerases that performs the resynthesis of DNA exhibit low-fidelity, but also because of the fact that the DNA sequence between the repeats is invariably lost, as is one of the two participating repeats.

Synthesis-dependent strand annealing (SDSA)

Similarly to DSBR, repair by SDSA is initiated by end resection in the vicinity of a DSB to provide 3'-single-stranded tails, then these 3'- free ends invade into a homologous sequence. The mechanism of SDSA, however, does not involve intermediates with Holliday junctions, but works by strand displacement, annealing of the extended single-strand end to the single-strand on the other break end, followed by DNA synthesis and ligation of the free ends [reviewed in 318]. SDSA results in non-crossover products and the region of the conversion is limited on both ends of the initial double-strand break.

Break-induced replication (BIR)

BIR is carried out when the double-strand breaks occur in DNA molecules currently undergoing replication. It generates entirely non-reciprocal crossovers, as a replication fork at the site of the free-end invasion may proceed with subsequent DNA synthesis up to the end of the replicated region – presumably, to the end of the chromosome [319].BIR may cause substitution of all DNA beyond the break site. When occurring at sites of microhomology (only several bp long), the mechanism of BIR may alter the genome in a quantitative as well as in qualitative manner, introducing at breakpoint junctions DNA sequences derived from elsewhere in the genome (microhomology-mediated BIR, MMBIR) [320,321]. On one hand, this would increase the number of copies in which a sequence is presented in the genome. On the other hand, it would produce discontinuity in genomic sequences, deleting certain regions, multiplicating others and translocating certain parts of

the genome to other locations, placing them in the 'correct' or the 'reverse' orientation to the direction of transcription of the recipient locus.

Fork stalling and template switching (FoSTeS) is a mechanism closely related to MMBIR. It is based on the capability of the replication fork to stall and switch templates using regions of microhomology in the template in order to anneal sequences and prime DNA replication [322,323]. The forks involved in FoSTeS may be close to each other or may be separated by considerable distances. The mechanism enables the joining of different sequences located in different positions in the genome and can result in complex non-reciprocal rearrangements, recurring as well as non-recurring.

3.3.2. Repair by non-homologous end joining (NHEJ)

Basically, broken ends may be rejoined by ligation after making sure that a free 3'-OH and a 5'-phosphate ends are available for joining. To achieve this, some nucleotides at the damage site may need to be excised and subsequently resynthesised. This does not require the presence of a homologous template, and is, therefore, known as non-homologous end joining (NHEJ).

The mechanism of repair by non-homologous end joining was initially believed to be almost exclusively reserved for eukaryotes, but later it became clear that bacteria may use it as well [162,324].

NHEJ usually uses short (1–10 bp long) homologous regions in the vicinity of a double-strand break in order to initiate DNA pairing and synthesis. Since DNA is built with only 4 building blocks, such regions of microhomology are normally not difficult to find. If, however, suitable homologous regions do not exist in the immediate vicinity of the DSB, the ends may be processed (elongated or shortened) in order to generate regions of sequence similarity, which then are aligned [325,326]. This leads to introduction of deletions in the final (joined) sequence. The mechanism involving recession of ends is sometimes called microhomology-mediated end joining (MMEJ). It is an alternative mechanism that is even more error-prone than 'classic' NHEJ. MMEJ employs the same Mre1-dependent mechanism for end resection as in homologous recombination [327].

In specific cases, the nonhomologous ends subject to joining may have added non-template nucleotides prior to joining. Such is, for example, the mechanism for increasing the diversity of repertoire of T-cell receptors and immunoglobulin genes during V(D)J recombination [328,329]. This requires the additional activity of terminal deoxynucleotidyl transferase [330].

NHEJ is an inherently error-prone mechanism. This is associated, on the other hand, with the associated loss or rewriting of the genetic information from the site surrounding the DSB [331]. To this adds up the error rate of the polymerases participating in the resynthesis of DNA (DNA polymerase λ or μ), which generally lack proofreading properties and are capable of adding non-template nucleotides [87,171]. However, since the eukaryotic genomes contain a significant amount of non-coding DNA, NHEJ is a viable option for repairing double-strand breaks, as the risk that a DSB would occur in a crucially important sequence is very small, and the risk that a DSB occurring anywhere in the genome would have adverse consequences is great. The error-proneness of NHEJ introduces a certain

amount of genetic variability, too, though it can produce potentially deleterious genomic rearrangements [171].

The final ligation of ends in NHEJ is carried out by the XRCC4/ DNA ligase IV complex [174]. The presence of an auxiliary factor, called NHEJ1(nonhomologous end-joining factor 1, also Cernunnos) is also needed. Cernunnos provides the physical connection between XRCC4 and the other NHEJ factors and allows XRCC4 access to the free DNA ends [332,333]. Defects in human genes coding for DNA ligase IV, Artemis and NHEJ1produce phenotypes of severe combined immune deficiency with radiosensitivity [332,334,335].

3.4. Translesion RNA and DNA synthesis

There are other rules, but you'll find out
what they are when you break them.
Sub-commander Raiker, Spacefall (Blake's 7), 1978

There may be times in the life of the cell when it is unconditionally needed that cells with damaged DNA survive at least until the current round of transcription and/or replication is complete. Replicative DNA polymerases and RNA polymerase II normally stall at sites of damage and signal to the cell repair machinery to assemble and repair the damage. Therefore, survival of cells with damaged DNA is usually achieved by employing polymerases that are capable of adding the 'correct' nucleotide opposite the damaged nucleotide and/or exhibit lower substrate specificity (recognising 'non-canonical' substrates') [182,336]. These polymerases are usually labelled 'low fidelity', but that is only partially associated with their error rate. Indeed, it is understandably higher than the error rate of 'nominal' transcription and replication polymerases. Therefore, translesion bypass mechanisms are inherently mutation-prone.

3.4.1. Translesion transcription

Translesion transcription is largely dependent on the type of damage present in the DNA template. RNA polymerases may recognise several different 'non-canonical' nucleotides in the DNA template and add the correct nucleotide, so that the transcript would carry the 'correct' sequence even though the template is damaged. Indeed, when uracil is present in DNA, RNA polymerases are capable of read-through transcription, inserting the 'correct' nucleotide against the altered site. When unrepaired AP-sites are present in the template, the RNA polymerase II may proceed with transcription, adding a random nucleotide (most commonly, A) opposite the AP-site. When the sequence of protein-coding genes contains oxidised nucleotides, however, e.g. 8-oxoguanine, the RNA polymerase II is usually stalled at the damage site, sending a recruitment signal to the TC-NER repair machinery [337,338].

3.4.2. Translesion replication

Translesion replication ensures that damaged sites in DNA are read-through during DNA-dependent DNA copying, thereby preserving the genome integrity. Thus, the cell may escape from apoptosis, complete the current replication cycle and sometimes may still be able to divide further. Even in case the damage persists and the DNA containing the altered site is replicated further, one of the two daughter molecules would have inherited the intact DNA strand, originating from the strand synthesised in translesion copying. True, the newly synthesised strand may potentially carry a mutation, due to the higher error rate of

translesion replication, but since translesion replication is normally used on short regions only, the risk of introduction of mutations is relatively low.

In prokaryotes, translesion replication is an adaptive mechanism for increasing the chance of survival in adverse environmental conditions (hence the commonly used term 'SOS response'). Also, bypass replication in bacteria has been found to be implicated in the development of resistance to quinolone antibiotics [339,340].

The SOS response pathway

SOS response is a prokaryotic mechanism for initiation of DNA repair that, depending on the type and the scale of the damage, may be routed towards repair by nucleotide excision or toward mutation-prone bypass replication of the damaged template. The major error-prone polymerases acting in the SOS response are the Y-family prokaryotic polymerases IV and V, but polymerase II may also be SOS-inducible [341]. The efficiency of error-prone translesion repair may be different depending on the type of damage and the polymerases involved [342].

Many prokaryotic genes coding for products acting in DNA repair contain a specific regulatory sequence in their 5'- untranslated region, termed an SOS-box. In unstressed cells, this sequence is normally bound by the repressor protein LexA. In the event of DNA damage, single-stranded DNA ends accumulate rapidly, which serves as a recruiting signal for the protein RecA [343]. Upon binding to single-stranded DNA ends, RecA is activated, stimulating the autoproteolytic properties of LexA [344]. As LexA is degraded, the SOS-boxes (previously protected by LexA) are eventually made available for binding of transcription factors. This results in the expression of about 30 different genes, among which are the proteins of the Uvr (NER) repair pathway. Thus, the activation of the SOS response at first level directly activates repair by nucleotide excision.

If it turns out that the damage is far too severe or extensive that the mechanism of NER cannot manage it, the prokaryotic cell may die, as the cell cycle is halted by default until the damage is repaired. In this case, the mechanism of autoproteolysis described above causes the levels of LexA to fall even lower, which eventually causes release of LexA bound to regulatory sites of other SOS-box specific proteins, whose affinity for LexA is higher than the affinity of the NER repair genes. Among these 'second level' genes are, for example, the genes coding for the proteins umuD and umuC. These two form an enzymatic complex (polymerase V, sometimes also called "mutasome"), containing 2 subunits of umuD, one subunit of umuC and several RecA molecules [345]. RecA aids in the displacement of the stalled DNA polymerase from the DNA at the site of damage and exchanges it with the umuD2umuCcomplex, which carries out the bypass replication in the region containing the damage (Fig. 10).

As soon as an undamaged DNA region is reached, the umuD2umuC complex is exchanged again for the 'nominal' replicative DNA polymerase (usually PolIII).

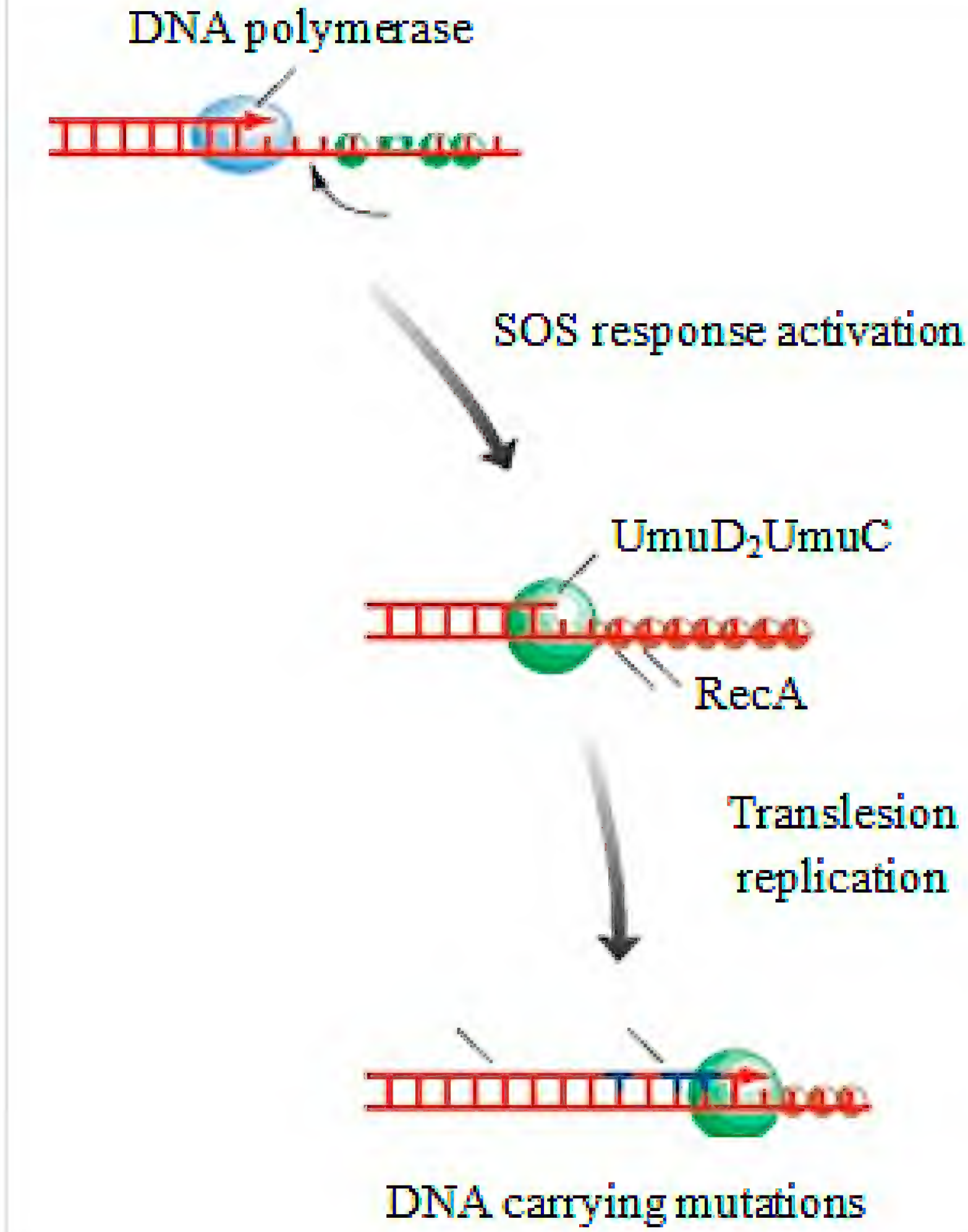

Figure 10. Translesion replication in SOS response, involving the UmuD2UmuC complex (DNA polymerase V).

Apart from the SOS response pathway, several other mechanisms for translesion replication are currently known. They generally involve error-prone polymerases other than polymerase V – specifically, polymerases IV and II. All three polymerases (PolII, PolIV and PolV), however, may be induced by the SOS pathway, but may work on templates with different types of damage. Bacterial DNA polymerase II, for example, may carry out read-through replication in templates with DNA adducts, while polymerase IV readily replicates DNA containing mismatched nucleotides [346,347].

Some authors believe that translesion replication in eukaryotic cells is a specific mechanism designed to increase the adaptivity through mutagenesis, stimulating the evolutionary processes [348,349].

4. Inherited diseases associated with defective DNA repair and maintenance of genome integrity

Tell me, silvery Moon,
What are you going to do with a child made of flesh?
Mecano, Hijo de la luna (Entre el cielo y el suelo, 1986)

Congenital deficiencies of DNA repair are associated with many pathological phenotypes. Some of these are early-onset and severe, others may be mild or subclinical and manifest relatively late in life. Many of the genes, molecular defects in which are associated with defective DNA repair, have been unequivocally identified and the genotype-phenotype correlations have been established, while others are still under investigations as candidate genes.

Historically, the first to be ever recognised and described as 'diseases of repair' were the gross defects of nucleotide excision repair, producing severe early-onset disease phenotypes – xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichotiodystrophy (TTD). Not much later, the underlying genetic defects causing some of the severe combined immune deficiencies in man were identified as a result from defects of DNA repair by recombination. This was followed by recognition of diseases and conditions, caused by defects in genes coding for proteins implicated in the maintenance of the genome integrity (ataxia-telangiectasia, Li-Fraumeni syndrome, Werner syndrome, Bloom syndrome, hereditary breast and ovarian cancers, and others) and mismatch repair (some types of familial colorectal cancer). The existence of diseases of base excision repair was unknown for quite a long time. It was universally believed that the defects in BER genes were associated with such severe phenotypes, that presumably all of the affected foetuses died in utero, so that the associated conditions never came to clinical attention. It was not until several years ago that it became clear that inherited defects of BER could be compatible with life well into adulthood, but they tended to be associated with predisposition to multifactorial diseases and conditions (e.g. diabetes type 2, etc.) rather than specific monogenic diseases. Approximately at the same time, genes, mutations in which were known to be associated with typical monogenic diseases of NER, were proven to be implicated in the pathogenesis of age-related multifactorial disease as well (see below). At present, it is believed that defects and polymorphic variants in DNA repair genes may play roles in the pathogenesis of virtually all types of human disease, as well as in the response to different therapies and the risk for various adverse reactions to specific treatments.

4.1. Human diseases and conditions associated with defects in NER

This category comprises all seven complementation groups of xeroderma pigmentosum (XP); XP variant form; Cockayne syndrome (type A and B) and the three types of trichotiodystrophy. Defects in the some of the NER genes (e.g. XPB, XPD) may produce any of the three disease phenotypes, while defects in other genes (e.g. XPC) result in a defined phenotype. There may be traits (sometimes, syndromic traits) shared between different disease phenotypes. For example, the syndrome of DeSanctis-Cacchione may be seen in patients with defects in different XP-associated genes (*XPA*, *XPD*, possibly any of the other

XP genes, but is most commonly seen in patients carrying mutations in the *XPA* gene) and also in patients with mutations in the *CSB* gene (usually associated with Cockayne syndrome type B) [350–352].

There may be mixed forms between XP and CS. Sometimes, a mutation in a gene associated with XP may produce a non-XP phenotype (e.g. mutations in the *XPD* gene may cause, besides XP, CS, TTD and mixed XP-CS states, cerebrooculofacial syndrome type 2 (COFS2). Below we will go into more detail about the complicated genotype-phenotype correlations between diseases of NER, but at present we will offer the simplest criterion for distinction between different NER diseases and elaborate later. Generally, Cockayne syndrome is associated with defects in transcription-coupled repair, while XP (except XP variant) and TTD are caused by mutations that affect global genome repair as well. Different forms of XP may be associated with defects in both types of NER (complementation groups -A, -B, -D, -E, -F, -G); or in global genome repair only (XP-C); or in or in translesion DNA synthesis (XP variant, XP-V). In TTD, besides abnormalities in DNA repair, the general mechanism of transcription is affected as well [353,354].

This classification is only provisional. As the genes coding for CS proteins play a role not only in NER, but in the maintenance of genome integrity as well, CS may be classed together with diseases caused by inadequate control of genome integrity (A-T, Werner and Bloom syndromes, etc.). TTD in this case must be treated as a separate type of disease, as disordered transcription plays as crucial a role for establishment of TTD phenotype as disordered DNA repair.

4.1.1. Xeroderma pigmentosum

Men shut their doors against a setting sun.

William Shakespeare, Timon of Athens (1623),

Act I, Scene II.

The first major breakthrough in the research of mechanisms of nucleotide excision repair occurred in the late 60-ties of the XX century, when Cleaver discovered that NER was defective in a group of patients suffering from a rare genetic disease characterised by UV-sensitivity, pronounced cancer-proneness, neurological abnormalities and dry and pigmented skin. The latter gave the name of the disease – xeroderma pigmentosum (XP).

XP is inherited by autosomal recessive model, that is, both copies of the responsible gene must be defective in order to produce a disease phenotype. Thus, affected children are usually born to clinically healthy carrier parents. The incidence of XP is estimated at 1:200,000–1:500,000 live births in different European and American populations, on average 1:250,000. For reasons still unknown, the prevalence of XP is about 6–10 times higher in Japan (roughly 1:40,000) [2,355]. The Navajo reservation in the United States reportedly has a striking □1:30,000 incidence of XP. In both cases, the most likely explanations for the elevation of the relative number of children with XP are founder effects and interbreeding. This may also explain the fact that in Japan the most common form is XP-A, while the form most commonly seen elsewhere is XP-C.

Seven complementation groups and a variant from have been discriminated in XP. Each is associated with defects in different genes, coding for proteins acting in the same mechanism for DNA repair by NER, except the variant form, which is associated with defects

in one of the translesion DNA polymerases that repair photoproducts in the skin (see below).

The XP phenotype (except in XP variant) arises as a result of inherited molecular defects in genes coding for proteins needed in the early stages of NER repair (damage recognition – *XPA*, *XPC*; DNA unwinding – *XPB*, *XPD*; proteins binding to damaged DNA –*DDB2*; and introduction of excisions around the damage site – *XPF*, *XPG*). Sometimes, de novo mutagenesis may be responsible for occurrence of XP. Genes coding for products associated with NER repair are usually highly conserved, therefore, a 'hit' almost anywhere in the coding sequence would produce a XP-associated phenotype [3]. The phenotype of each complementation group may have one or many shared features with the phenotypes produced by XP of other complementation groups.

A unifying feature of all forms of XP is sensitivity to UV irradiation. In individuals with XP even very small doses of UV (for example, the negligible amount of UV emitted by incandescent lamps or the unfiltered residue passing through windowpanes made of ordinary glass) may have adverse consequences and the damage is cumulative. UV is virtually unavoidable during the day and complete anti-UV barrier protection is very difficult to achieve. Unless specific and sustained effort is made to protect the individual with XP from daylight and artificial sources of UV, XP follows a severely progressive course and affected individuals that have not received any or have received minimal treatment rarely live into the second or third decade of their lives, usually dying of skin cancer or, less often, of other type of XP-associated tumours.

Usually, it is the extreme UV-sensitivity that attracts the attention of parents and physicians in a XP-affected child. It manifests with disproportionately severe sunburn after minor UV exposure and/or early appearance of dysplastic skin changes, which may quickly progress to overt skin cancer. In individuals with XP the risk of skin cancer (non-melanoma and melanoma) is elevated about 1000 times compared to the population risk. First skin tumours in XP usually appear in childhood (on the average, around the eighth year of age). For comparison, the risk for the average clinically healthy person to develop skin cancer is between 1:200 and 1:500, and tumours very rarely appear before the third or fourth decade. XP patients are at an increased risk of various non-cutaneous tumours as well, including different types of leukemia, astrocytoma, medulloblastoma and brain sarcoma, and also pancreatic, testicular and gastric cancer [356]. The age at onset of internal tumours in XP patients is earlier than in the non-XP population, but may well extend into the fifth and sixth decade of life, that is, much later than UV-related skin cancers and close to the age when the risk for carcinogenesis becomes higher in the clinically healthy individuals. Obviously, the primary health concern in people living with XP is the UV-related carcinogenesis, as it may strike much earlier than other types of tumours.

The course and the severity of XP may vary greatly between different patients. Even within a group of patients carrying defects of the same complementation group, the phenotype may vary from patient to patient depending on the type and the precise location of the particular molecular defect, as well as additional genetic (e.g. carriership of polymorphisms in other genes implicated in DNA repair) and environmental factors (e.g. onset, efficiency and regularity of sunscreening). Also, the phenotype of the individuals within a single

complementation group may vary because of the codominance effect (that is, each of the two disease alleles may have its own weight in the constitution of the disease phenotype) [357]. As a rule, when XP-associated mutations affect genes coding for products acting in the earliest stages of NER – damage recognition (XPA) or unwinding of DNA at the damage site, providing access of the cellular repair machinery to the site of damage – (the helicases XPB and XPD), the resultant phenotype is more severe, usually with accompanying neurological abnormalities manifesting early in life, even in the neonatal period. An apparent exception is XP-C, in which neurological deficits are very uncommon and the age on onset is in later childhood (see below). When the defects are in genes coding for proteins acting in later stages of NER (e.g. the endonucleases XPF and XPG), the associated phenotype may be milder, with predominant UV-sensitivity features. Neurological abnormalities may appear later, if at all (Table 2).

Mutations in damage-binding protein 2 (DDB2) are responsible for XP-E [358]. DDB2, together with the related DDB1 protein are components of the UV-damaged DNA-binding protein complex (UV-DDB) [359,360]. It recognises UV-induced DNA damage (cyclobutane pyrimidine dimers, 6-4 photoproducts) as well as abasic sites and nucleotide mismatches [361,362]. The associated XP-E genotype is usually limited to photosensitivity only (Table 2).

Table 2. Genotype-phenotype correlations in xeroderma pigmentosum

Gene/locus	XP complementation group or other associated condition/s
XPA 9q22.33	Xeroderma pigmentosum complementation group A (XP-A)–extremely photosensitive, cancer-prone, with early-onset neurological abnormalities – most common form of XP in Japan, elsewhere relatively rare. Or: De Sanctis-Cacchione syndrome (may be seen in XP-A and XP-D (possibly in other forms), and in Cockayne syndrome type B) [352]. When in the course of XP-A, photosensitivity and cancer-proneness add up to the features of DeSanctis-Cacchione syndrome, though the cutaneous symptoms may not be as severe as in 'classic' XP-A [350].
XPB 2q14.3	Xeroderma pigmentosum complementation group B (XP-B) – very photosensitive, cancer-prone, likely with early-onset neurological abnormalities – a rare type. Or: XP/CS [363]. Or: Trichotiodystrophy [363]
XPC 3p25.1.	Xeroderma pigmentosum complementation group C – the most common type (about 1/3 of all patients), usually without neurological symptoms [364] (for more details, see the text).
XPD 19q13.32	Xeroderma pigmentosum complementation group D (XP-D)– very photosensitive, cancer-prone, likely with early-onset neurological abnormalities – relatively common, about 15% of all cases. Or: XP/CS. Or: De Sanctis-Cacchione syndrome. Or: Trichotiodystrophy [365–367]. Or: COFS2 [368]
DDB2 11p11.2	Xeroderma pigmentosum complementation group E– extremely rare, only several cases described worldwide. Usually manifests with UV-sensitivity only [369,370].
ERCC4 16p13.12	Xeroderma pigmentosum complementation group F– relatively uncommon, about 6% of all patients. The resultant phenotype may be heterogeneous,

	with UV-sensitivity only or with mild neurological abnormalities, manifesting at later age [371,372].
ERCC5 13q33.1	Xeroderma pigmentosum complementation group G - relatively uncommon, about 6% of all patients. Manifests with photosensitivity, although the cutaneous presentations are usually relatively mild. May be accompanied by neurological abnormalities, congenital cataracts and mental retardation. Extremely rare, with only 16 cases described worldwide [373]. Or: XP/CS
POLH (Polymerase η) 6p21.1	Xeroderma pigmentosum variant– very common, about 20% of all XP patients NER is essentially intact, translesion DNA synthesis is affected [374].

Note: The COFS3 phenotype [375] is now included in XP-G.

The variant form of xeroderma pigmentosum (XP-V) is not associated with defective NER. The molecular defects in XP-V affect the gene coding for the translesion polymerase eta (*Polη*), which copies UV-damaged DNA templates with relatively high fidelity, adding the 'correct' nucleotide opposite photoproducts in DNA (e.g. two As against a thymine dimer, etc.) [184].

In the absence of functional polymerase eta, the translesion repair is taken over by the lower-fidelity polymerases zeta and iota, resulting in accumulation of UV-induced damage [181,184]. Polymerase iota, a member of the X family of error-prone polymerases (related to terminal transferase) is capable of adding nucleotides during copying damaged templates without much discrimination of whether the nucleotide is a match or not [87]. Polymerase zeta elongates the resulting partially double-stranded regions containing mismatches [181]. Since the error rate of the polymerases iota and zeta is higher than the error rate of polymerase eta, there is a risk for cancerous transformation of skin cells in XP-V, although not that high as in 'classic' XP. The major symptom of XP-V is photosensitivity and cancers appear much later than in 'classic' XP (between 15 and 40 years of age). There are no associated neurological abnormalities or congenital malformations.

Xeroderma pigmentosum of complementation group C is associated with mutations in the gene coding for the earliest protein recognising the damage in global genome repair, XPC. The prevalence of XPC compared to the incidence of other forms of XP is very high, about over 30% of all cases, except in some populations where founder effects are suspected. XP-V is the second most common XP form, about 20% of all patients. The unusually high prevalence of XP-C and XP-V may be related to a variety of reasons. The molecular defect in XP-C does not affect all types of NER. Damage in transcribed genes is promptly repaired in individuals with XP-C, as presence of damage is recognised by a XPC-independent mechanism. As genes are switched on and off during postnatal development, much of the damage is repaired in its own time. The only tissue that is crucially dependent on GGR is the skin, as its cells are attacked daily by genotoxic UV light. Therefore, the incidence of cancer in XP-C is as high as in the more severe phenotypes of XP-A, -B and -D, where both types of NER are affected. XP-V cells are NER-proficient altogether and deficient in translesion DNA synthesis. Since translesion replication is an emergency mechanism, it is only significant in tissues that are directly exposed to genotoxic attack since the day of birth – again, the skin.

Only a small proportion of embryos and foetuses with any type of genetic defect survive long enough in utero to be born alive and live through the early postnatal period [376–378]. In general, the chances that a foetus affected with genetic disease would survive to term are inversely associated with the severity of the molecular defect and/or the severity of the resultant phenotype, and/or additional factors (e.g. environmental). Embryos are well protected from UV inside the mother's womb. Presumably, the amount of damage which needs to be repaired by GGR in the embryo and the foetus is not that large, allowing a significant part of XP-C foetuses to live to term and be born after an uneventful pregnancy. Same goes for XP-V, in which the need for translesion replication in the skin only emerges after the baby has been born. In the other major forms of XP (-A, -B, -D), the underlying defect is more severe, affecting TC-NER as well. This may effectively preclude the survival of the majority of the affected foetuses to gestation age that is advanced enough to ensure perinatal and postnatal survival. From this point of view, GGR and translesion replication are at least partially expendable, while TC-NER is not. XP is altogether more common than the other two diseases related to defects in NER (Cockayne syndrome and TTD). This may be related to the fact that foetuses affected with diseases of TC-NER rarely survive to term at all. Apparently, deficiencies of GGR and translesion replication are compatible with life. The molecular defects in XP-C and XP-V are, however, severe enough to decrease the quality of life for the patients and shorten their lifespan compared to clinically healthy people (that is, in absence of adequate and systematic treatment).

The discovery of the rodent repairadox [22,239] provided a definite proof that some higher animals (specifically, rodents) may rely mostly on TC-NER for repair of DNA damage, selectively inhibiting repair in untranscribed DNA. Rodents, however, have a naturally short lifespan. They rarely live beyond 4–5 years (ordinary mice and rats – about 2–3 years), while clinically healthy humans of today generally live 7–8 decades and beyond. Rodents and humans are relatively closely related in evolutionary and genetic aspects and have genomes of approximately the same size, containing similar amounts of untranscribed DNA. Only short-lived species, however, may afford to dismiss GGR altogether, as the cumulative effects of unrepaired damage in the untranscribed regions would not become manifest before a significant amount of time has passed anyway. In the absence of functional GGR, unrepaired damage in untranscribed regions may slowly accumulate over several years, becoming significant enough so as to cause visible abnormalities or cancer only after this time is exceeded. Indeed, individuals with XP-C rarely become symptomatic before their 5^{th} or 6^{th} year of life, while those with XP-A, -B and -D may be brought to clinical attention much earlier, sometimes immediately after birth – usually because of the associated neurological deficits and various inborn anomalies.

The case might be the same with defects in the gene coding for the partner of XPC in recognition of the damage, *RAD23B*. It localises to 9q31.2 [379]. Currently there is no known human disease or condition associated with mutations in this gene. In murine models, it has been shown that knockout mouse embryos completely deficient in hHR23B have low survival rates, most of them dying in utero or shortly after birth. The surviving Rad23B-null mutant mice showed high incidence of developmental abnormalities, stunted growth and male sterility [380]. Cultured fibroblasts from knockout Rad23B-deficient

embryos, however, did not exhibit UV-hypersensitivity. It was proposed that Rad23A, homologue of Rad23B, was capable of taking over most of its repair-related functions, but none of its functions in embryonic and postnatal development [380]. It is possible that the human equivalent/s of RAD23B-deficiency related disorders are not recognised yet as such because of the low survival rate of affected embryos and/or the severe malformations in the newborn, which may resemble other disorders of the large and heterogeneous groups of congenital anomalies.

Neurological damage is considered a very unusual feature in patients with XP-C. In fact, the presence of neurological symptoms is a serious basis for reassessment of diagnosis. The fact that XP-C affected individuals are usually neurologically spared might also be explained from the viewpoint of prioritisation of NER repair in transcribed and untranscribed genomic regions.

While XP is almost always associated with drastically increased risk for cancer (especially skin cancer), the other two diseases of NER (CS and TTD) usually are not (unless it is the severe 'mixed' phenotype of XP-CS). XP patients are photosensitive, as a rule, but may or may not exhibit neurological abnormalities. Patients with Cockayne syndrome and trichotiodystrophy are usually photosensitive and may be neurologically impaired, but are not cancer-prone. Cockayne syndrome is usually associated with profound neurological pathology and adipose tissue degeneration while TTD patients usually manifest predominantly with skin abnormalities and brittle hair and nail. CS and TTD affect more organs and systems, while XP appears to affect primarily the skin (as UV penetrates only 1–2 millimetres within the skin) and, rarely, the nervous system. The clinical features of all three major diseases of NER may intermingle and defects in the same genes may produce clinical phenotypes of XP, CS or TTD, or mixed states (Fig. 11). For example, mutations in the *XPB* or the *XPD* gene may produce XP of complementation groups B (XP-B) or D (XP-D), respectively; but also CS; TTD, or mixed XP-CS, XP-TTD or CS-TTD states, despite the fact that the affected gene is the same [367,381].

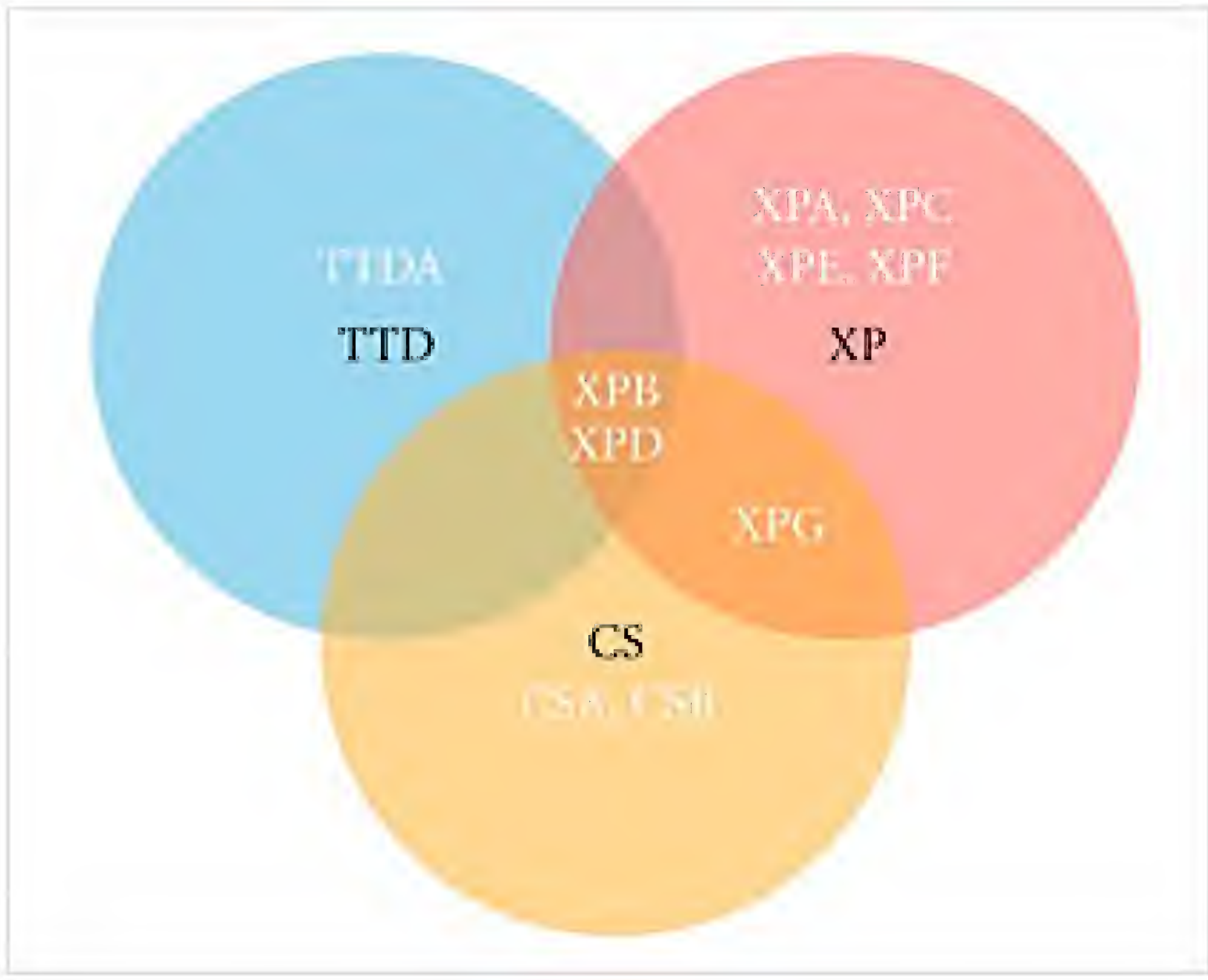

Figure 11. Overlapping of clinical forms of the three major diseases of NER.

Some of the symptoms of XP (UV sensitivity and the associated susceptibility to skin cancer) may be greatly ameliorated by treatments targeted at minimising UV exposure and augmenting the residual DNA repair capacity. Since barrier protection from UV exposure is crucial for patients with XP from early age, extended child-parent networks exist worldwide for families with XP-affected members. The main efforts are directed towards providing accessible UV- safe environments in which affected children and adults may receive adequate education (respectively, to provide opportunities for jobs for individuals with XP) and encourage socialisation within and outside the community, as well as raising the social awareness about XP. Prominent among these organisations are, for example, Les Enfants de la lune Xeroderma Pigmentosum Association at the European organisation of rare diseases and the Xeroderma Pigmentosum Society in USA (XPS). The activities of these societies are centred around promotion and encouragement of night-time activities (as night is, naturally, the safest time for individuals with XP to go out in the open with minimal risk of inadvertent UV irradiation), including building and maintenance of 'night camps' (e.g. Camp Sundown in Poughkeepsie, NY). Also, the XP societies provide support for families for reversion of the day/night cycle, popularisation of anti-UV measures (including safe and cheap ideas to minimise the daily UV dose), increase the watchfulness for early signs of disease associated with UV exposure and the available therapeutic options.

Modern therapeutic approaches for xeroderma pigmento¬sum are based primarily on prevention of UV irradiation and minimisation of the daily UV dose (including modification of home environment), coupled with frequent regular monitoring of skin and mucosa for early identification of skin changes with suspect neoplastic potential; and protection and

treatment of skin zones that are dry and prone to pigmenting. This includes anti-UV covering on all windowpanes; installing minimal-UV lighting at home as well as in school; carrying UV-dosimeters all the time, so as to know when inadvertent exposure becomes too much; wearing UV-absorbing clothing (dark colours, several layers, hats and long hair to protect the skin of scalp and neck), as well as more unconventional protection such as anti-UV helmets, face shields and face masks, similar to the shield seen in Fig. 12.

Figure 12. Protective anti-UV face shield designed to be worn all the time when not sleeping. The design of the face shield is recommended by the association "Les enfants de la lune" and is approved by Eurordis. The patient wearing the face shield in the picture is one of the XP-affected twin boys Vincent and Thomas, of the French family Séris. Françoise and Bernard Séris have 3 other children which are unaffected by XP. The Séris family is among the founders of "Les enfants de la lune" and activists in the movement for introduction and popularisation of anti-UV measures for XP-affected families. (Eurordis Rare Diseases Europe, Xeroderma Pigmentosum – The Séris Family).

Normally, anti-UV treatments are based on a purely preventative approach and there is not much to be done after it has already happened. Barrier treatment is normally the staple approach in anti-UV strategies and is crucially important in the treatment and management of XP. Relatively recently, however, an additional therapeutic option was developed – namely, the post-irradiation treatment with preparations of T4 endonuclease V [382–384]. This approach is based on a different principle from conventional barrier protection, putting

to work those natural defences against DNA damage which are intact. It is designed for use after the UV damage has already occurred, as T4endoV works on preformed photoproducts in DNA, rather than preventing them from forming.

The working principle of the T4endoV-based method for anti-UV protection is quite simple. The enzyme is packed in appropriate delivery systems and applied to the skin. Usually, the enzyme preparation is integrated into skin ointments and lotions for topical application, similarly to manufacturing of barrier anti-UV lotions. The vehicles may be different, but generally gel formula or liposome suspensions are used. The latter seems to be more efficient, as liposomes penetrate the upper skin layers easily, dissolve readily upon contact with lipophilic cell membranes and release the active substance not on the skin surface (which is made of dead cells), but within the lower layers of the skin, where the cells are alive and actively dividing (that is, where the enzyme might be needed). The enzyme is usually recombinant, obtained by heterologous expression in prokaryotic cells [384,385]. T4endoV scans DNA for UV-damage (specifically, thymine dimers) and converts them into single-strand DNA breaks. While unrecognised thymine dimers may persist in DNA for quite some time (even in normal cells, but especially in cells deficient in NER), DNA breaks usually constitute a more potent signal to the repair machinery, causing recruitment of the repair proteins to the damage site. Of course, this will work efficiently enough if the amount of unrepaired thymine dimers in DNA is not extremely large. Therefore, this type of anti-UV protection cannot be used on its own, but only as adjuvant to the barrier methods – e.g. to make sure that even though the barrier protection might occasionally fail, the residual photoproducts would be promptly identified and processed.

It must be noted that conventional (barrier) methods of anti-UV protection and post-irradiation measures based on T4endoV and other repair enzymes (e.g. photoproduct photolyase from Anacystis nidulans) are fully compatible with one another, as their working principles are entirely independent [386,387]. Thus, provided that barrier protection is applied before the anticipated exposure and the T4endoV is applied directly after, the amount of residual skin damage would be reduced to the possible minimum. A combined anti-UV strategy has been proposed, equally applicable in individuals with UV-sensitivities of various origin and in clinically healthy people wishing to minimise the potentially harmful or unpleasant (erythema, pain, polymorphic light eruption, etc.) effects of excessive UV-irradiation and for [200, 201, 386].

4.1.2. Cockayne syndrome

Cockayne syndrome is a severe congenital condition, diagnosed in infancy or, sometimes, at birth, although late onset-cases have been recorded [388]. It is characterised by failure to thrive, severe neurological damage, mental retardation, premature loss of adipose tissue, early cataracts, retinal atrophy, sterility, tooth enamel abnormalities and photosensitivity without cancer-proneness. Cockayne syndrome falls into the category of segmental progeroid syndromes. Affected babies rarely survive childhood, but may live into teen age. Occasionally, in late-onset cases, some patients may live beyond the second or third decade of life [389]. As with most segmental progeroid syndromes, CS is inherited by autosomal recessive pattern, that is, affected children are usually born to asymptomatic carrier parents.

The CS phenotype results from decoupling of transcription and DNA repair [2,390]. In CS cells, the presence of stalled RNA polymerase II at sites of damage does not induce immediate recruitment of cellular NER machinery to actively transcribed genes. Repair of oxidation products, such as 8-oxoguanin, is also affected [353,354]. As oxidative damage is the primary type of damage in neural cells, the nervous system is a primary target in Cockayne syndrome. As ageing is closely associated with accumulation of oxidative damage, cells and tissues age at an accelerated rate in CS. As the underlying pathology of Cockayne syndrome affects repair of all actively transcribed genes in all cells and tissues, more tissues and organs are affected in CS in comparison with XP.

Depending on the affected gene (*CSA* or *CSB*), Cockayne syndrome is subdivided into two types – CS type A and type B. The two forms produce essentially similar phenotypes, with the difference that patients with CS type A may have disproportionately long limbs with large hands and feet. DeSanctis-Cacchione syndrome may be seen only in CS type B.

The *CSB* (*ERCC6*) gene (10q11) encodes a DNA-dependent ATP-ase, acting in the displacement of stalled RNA polymerase II from the site of the DNA damage [225]. Upon coming into contact with DNA, a dimer of CSB induces wrapping of the DNA fragment containing the lesion around the protein in an ATP-dependent process, thereby modifying the contacts surface between the DNA and the stalled RNA polymerase II [391,392]. CSB binding to DNA also serves as a recruitment signal for DNA repair machinery and histone acetyltransferase. Acetylation of histone protein decreases their positive charge, thereby decreasing their affinity to DNA, resulting in relaxation of the chromatin structure in the vicinity of the modification [reviewed in 393].

The *CSA* (*ERCC8*) gene (5q12.1) codes for a protein recruiting the nucleosomal binding protein HMGN1, the transcription elongation factor complex TCEA1/TFIIS, and XAB2 (XPA-binding protein 2) to the site of RNA polymerase II-blockage, inducing initiation of repair of the lesion and subsequent recovery of transcription [392,394, 395]. CSA promotes the degradation of CSB by the ubiquitin-dependent mechanism, which is essential for the recovery of RNA synthesis after TC-NER has been completed [392,395]. CSA and CSB proteins interact closely with TFIIH [251,396, 397].

The incidence of Cockayne syndrome worldwide is about 1:370,000 live births [355]. About 80% of all patients with Cockayne syndrome have mutations in the *CSB* gene, while in the remaining 20% the mutation is in the *CSA* gene. Some mutations in XP genes (e.g. *ERCC3 Phe99Ser*; *ERCC5 2972T*; *ERCC3 IVS14ASC-A*; *ERCC2 D681H*, etc.) in *XPB* (*ERCC3*), *XPG* (*ERCC5*) and *XPD* (*ERCC2*) genes may produce mixed XP-CS states [398–402].

The first symptoms of CS usually appear early in life and the patients are severely affected. In 2004, however, Horibata et al. published a case report about homozygous carriership of a null CSB mutation in an adult male without any severe symptoms [403]. The first paper describing this phenomenon was actually published by Fujiwara et al. 23 years earlier (in 1981) and described the same patient at younger age [404]. Later, it was followed by several more reports, including the follow-up on the first case from childhood to adult age (Horibata's report) and a description of another patient with the same R77X nonsense mutation and a similar mild clinical presentation [405].

The phenotype described by the groups of Fujiwara and Horibata was of a normal boy and, later, of a normal adult male. The only abnormal feature, which brought the patient to clinical attention in the first place, was UV-sensitivity with freckling on sun-exposed areas, but it was mild enough not to cause serious trouble, except sunburns after minor exposure. Intelligence was normal on repeated testing. Neurological abnormalities were not identified at any age. The patient was taller than the population average (a very unusual feature, as CS is usually accompanied by disordered growth of long bones, producing short stature). The index patient had been in regular follow-up since age 8, when he was referred to the clinic because parents noticed his proneness to sunburn. At the age of 33, repeated testing did not found any gross pathology apart from the slight but measurable delay in the recovery of RNA synthesis in skin fibroblasts after UV irradiation. Genetic tests revealed the same nonsense mutation (R77X) in both CSB copies in the patient, producing 'null' alleles. The parents of this patient were first cousins, carrying the same rare familial mutation in heterozygous state. The R77X mutation converts a triplet coding for an arginine residue at position 77 in the protein to a termination codon, producing a truncated polypeptide, containing only the amino terminus of the CSB protein and only 76 amino acid residues in length. The normal ERCC6 protein is 1493 AA long. Therefore, the presence of a severely truncated protein can hardly account for the mild phenotype produced by the R77X mutation. The levels of the mutant protein in the patient were undetectably low, and whether the protein was expressed at all could not be verified. The mild phenotype of the patient could not be explained satisfactorily at the time and some kind of substituting mechanism that could potentially take over most of the functions of CSB in cases of complete absence of functional protein was proposed.

In 2012, a new gene has been identified (*UVSSA*), mutations in which were reported to cause UV-sensitive syndrome with features, similar to the mild UV-sensitivity phenotype in patients homozygous for the *ERCC6* R77X mutation [406]. UV-sensitivity related to defects in the *UVSSA* gene is listed in the Online Mendelian Inheritance in man (OMIM) database of human genetic disease as 'UV hypersensitivity syndrome 3'. The UVSSA protein has recently been found to stabilise the ERCC6 complex and to facilitate the ubiquitination of RNA polymerase II stalled at the sites of damage [407], so it may play a role in the amelioration of the phenotype, though the mechanism is still unclear.

Currently, OMIM contains more than one entry for CSB-associated monogenic disease, besides Cockayne syndrome type B – 'UV hypersensitivity syndrome 1' and cerebrooculofacioskeletal syndrome type 1 (COFS1). COFS1 is progressive neurodegenerative disorder, manifesting by microcephaly, congenital cataracts, mental retardation, and bone abnormalities [389,reviewed in 408]. It is transmitted by autosomal recessive pattern.

Inherited polymorphisms in the CSB gene may be associated with predisposition to multifactorial late-onset disease. For example, the 6530C-G variant in the 5'-region of *ERCC6* gene was found to be associated with age-related macular degeneration and increased risk for lung cancer [409, 410].

Defects in the *ERCC8* gene may also produce mild photosensitivity and freckling only without any other abnormalities – 'UV hypersensitivity syndrome 2' [411]. The authors

speculate that this rare phenotype is related to differential role of CS proteins in TC-NER and response to oxidative stress, as neurodegenerative changes and accelerated ageing are believed to be outcomes specifically of increased oxidative damage to DNA.

It is complementary, my dear Watson – how the results from the sequencing of the genome of J. D. Watson showed that whole-genome sequencing was still far from accurate

Notably, a presumed homozygous mutation in the *ERCC6* gene was initially reported in the list of single-nucleotide polymorphisms (SNPs) found during the sequencing of the genome of James D. Watson, one of the discoverers of the structure of DNA and Nobel laureate [412]. Specifically, the researchers found ≈9000 non-synonymous changes in known SNPs and ≈1500 in novel SNPs. After comparing the non-synonymous known SNPs with the Human Gene Mutation Database (HGMD) [413, 414]; thirty-two were found to match known mutations listed in HGMD. Twelve of the mutations affected loci where homozygous recessive alleles were known to cause to disease or at least a recognisable phenotype, and for the remaining 20 there were previously reported associations with increased risk for disease. Of the twelve disease-associated mutations, ten were identified as highly penetrant recessive disease-causing alleles. For three of these, only one allele per locus was identified – namely, in genes *ERCC6* (associated with Cockayne syndrome type B); *MYO7A* (associated with Usher syndrome 1B) and *PFKM*(glycogen storage disease VII). The SNP at the PFKM locus was typed with genomic coverage less than 5, but the allelic variant found was previously reported to produce a benign, yet clearly recognisable phenotype. The other two disease SNPs, however, were typed with coverage greater than 5, and yet only one allele per locus was found. This could be interpreted either as an experimental error, or the allele variant/s actually being in homozygous state. The experimental subject James D. Watson, however, who was 80 years old at the time of sequencing of his genome and fit as ever, had never shown signs of any of the three associated disease phenotypes. The authors of the 2008 paper proposed that he might be actually heterozygous for the disease mutations at these three loci (and, very likely, for many of the other SNPs which typed as homozygous), but that the 'normal' allele has been 'missed' ('dropped') during the process of sequencing [412]. Since some mutations in the *ERCC6* gene have been previously shown to produce only mild phenotypes, however, it would be interesting to see whether the results of re-sequencing of the genome of J. D. Watson would yield the same results as the first round of sequencing. If we accept, however, that the three homozygous variants that were reported were actually in heterozygous state, then heterozygocity for mutations that may potentially cause serious disease has been found for 10 loci in James D. Watson. This is higher than the average estimate of heterozygous carriership of 'lethal equivalents' in healthy individuals (believed to be more than 1.5–2 but definitely less than 10 [415, 416].

4.1.3. Trichotiodystrophy

Trichotiodystrophy (TTD) is a collective term used to describe three 'classic' UV sensitivity/brittle hair/brittle nails syndromes, and a fourth form, which does not include UV-sensitivity. 'Classic' forms of TTD are caused by mutations in three different genes. Two of these genes are the already mentioned DNA helicases XPB (2q14.3) and XPD (19q13.32) and the third is TDDA (GTF2H5) (6q25.3). All three are components of the transcription factor TFIIH [268,363, 417, 418]. TTDA stimulates the ATP-dependent helicase activity of

XPB, thereby facilitating the unwinding of DNA for easier access of the repair machinery [419].

The 'classic' forms of TTD are characterised by growth retardation, UV-sensitivity, dry ichthyotic skin, hair and nail abnormalities, mental retardation and sterility, with no significant elevation of the risk of UV-related skin cancer. In some patients with TTD, the associated features may be exacerbated during episodes of fever – for example, they may experience excessive hair loss during banal infections accompanied by fever [420]. It has been found to be associated with mutant thermolabile forms of XPD [421].

The fourth, non-photosensitive form of TTD (TTDN, hair/brain syndrome) is characterised by brittle hair, mental retardation, short stature and decreased male fertility, but no hypersensitivity to UV [422]. It is associated with mutations in the *C7ORF11* gene (7p14.1). The function of the C7ORF11 protein is still not entirely clear. It is known, however, to localise to the nucleus and is expressed in foetal hair follicles, as well as in the brain [423].

It has been proposed that trichotiodystrophy is an expression of a major disorder of transcription. In many TTD patients, genes coding for products that are not directly involved in DNA repair may not function normally. For example, reduced levels of beta-globin mRNA as well as protein were observed in TTD patients that had not any identifiable pathology in the beta-globin cluster or it control region [424]. TTD is sometimes classed as a separate disease entity from other 'repair diseases' (XP, CS), due to its being a 'transcription disease' [425].

The incidence of trichotiodystrophy is about 1:600,000 live births [355]. It is 1.5 times rarer than CS and more than 2 times rarer than XP. This could be expected, as the defect in TTD is, in molecular aspect, more profound than the defects, associated with XP and CS. Presumably, disordered basal transcription associated with TTD is more likely to cause early foetal loss in pregnancy than defects in DNA repair, which may, albeit rarely, produce viable newborns.

4.2. Human diseases and conditions associated with defects in repair by recombination

Inherited genetic defects in repair of double-strand breaks (by homologous recombination as well as non-homologous end joining mechanisms) in man are associated with various immune deficiency/radiosensitivity states, with or without neurological involvement [426, 427]. Mutations in genes, coding for products acting in repair of strand breaks may directly cause monogenic disease, or may constitute a genetic determinant in the predisposition to some multifactorial diseases and conditions. Among the former are some combined immune deficiency/ radiosensitivity syndromes with neurological involvement – e.g. RIDDLE syndrome, related to deficiency of RING finger protein (RNF168) [428], as well as phenotypes without immune deficiency – for example, defects in the *RAD50* gene produce a phenotype of Nijmegen breakage syndrome-like disorder, manifesting with microcephaly, mild psychomotor retardation, short stature and chromosome instability [174,426]. Among the multifactorial diseases associated with inherited defects in repair by recombination are some of the common autoimmune diseases (e.g. early-onset systemic lupus erythematosus) and some tumours, including childhood-onset malignancies [429, 430]. Carriership of mutations, associated with defective repair by recombination may affect the outcomes of

anticancer therapy, conferring higher risk for toxicity for some patients on conventional chemotherapeutic regimens [431].

4.2.1. Disorders of DNA repair associated with defective ligation of free DNA ends

Ligase IV is responsible for the end ligation step in repair of double-strand breaks by NHEJ, as well as in V(D)J recombination. Defects in *LIG4*, the human gene coding for DNA ligase IV, are associated with more than one disease phenotypes. One is characterised by developmental delay, immune deficiency and hypersensitivity to ionising radiation (LIG4 syndrome) [334]. Some of the cases of combined T cell-negative, B cell-negative, natural killer cell-positive severe combined immunodeficiency with sensitivity to ionising radiation (SCID-RS) are associated with mutations in the *LIG4* gene [432]. Some of the mutations in the *LIG4* gene, e.g. the Arg278His mutation allow for sufficient residual activity of the enzyme (5–10%), resulting in milder LIG4 syndrome phenotype. When co-inherited with other allelic variants in the *LIG4* gene, such as the Thr9Ile polymorphism, the effects seem to be additive, causing a severe phenotype in a patient carrying a mutation that is normally associated with mild LIG4 syndrome [433, 434]. What is truly interesting, however, is that carriership of the Thr9Ile polymorphism alone is associated with ***decreased*** risk for multiple myeloma, a tumour related to disordered immunoglobulin class switch recombination [433].

In a proportion of cases of T-cell leukemia, defects in the *LIG4* gene have been identified [435, 436].

Defects in the human gene coding for ligase III (*LIG3*, the ligase that restores the phosphodiester bond between the 3'-OH group of the newly synthesised DNA region and the adjacent 5'-phosphate of the 'old' strand in BER and NER) have not yet been found to be associated with any distinctive disease phenotype. Presumably, such embryos are too severely affected and die in utero. Experiments with mice, however, revealed that the disruption of the gene coding for ligase III in the nervous system resulted in profound mitochondrial dysfunction related to loss of mitochondrial DNA and severe ataxia. In the mouse heart, inactivation of ligase 3 resulted in mitochondrial dysfunction associated with heart failure [105].

Inherited defects in the gene, coding for DNA crosslink repair protein 1C (*DCLRE1C* (*Artemis*), 10p13) are associated with Omenn syndrome or severe combined immunodeficiency with radiosensitivity [335,437]. A somewhat similar phenotype (severe combined immunodeficiency, sensitivity to ionising radiation, microcephaly, and growth retardation) is associated with mutations in the gene coding for the auxiliary factor NHEJ1 (*Cernunnos*) [332]

4.2.2. Nijmegen breakage syndrome (NBS)

NBS is a monogenic disorder characterised by microcephaly with normal intelligence, short stature, specific 'bird-like' facies, combined immune deficiency, chromosome instability and propensity to haematological malignancies. Patients with NBS may experience severe toxicity following chemotherapy with radiomimetic agents and/or radiotherapy. Nijmegen syndrome is transmitted in autosomal recessive pattern [438].

The *NBN* (*NBS1*) gene is localised at 8q21.3 and encodes the protein NBS1 (nibrin), functioning in repair of double-strand DNA breaks and in activation of the p53-independent

pathway of induction of apoptosis in cells with irreparably damaged DNA [439, 440]. NBS1 is one of the components in the MRN protein complex (MRE11-RAD50-NBS1) that implements the initial recognition of double-strand breaks and the processing of free DNA ends in eukaryotic cells.

4.2.3. Segmental progeroid syndromes other than Cockayne syndrome (Bloom syndrome, Werner syndrome)

Gene mutations associated with deficiency of two of the proteins with helicase activity functioning in repair by homologous recombination (REC protein Q-like helicases, RECQL2 (WRN) and RECQL3 (BLM)) may produce segmental progeroid syndrome phenotypes. These proteins are homologous to the prokaryotic RecQ helicases and possess DNA-dependent ATP-ase, DNA helicase and 3'-to 5'- single-stranded DNA translocation activities [153, summarised in 441]. Molecular defects in the gene coding for RECQL2 result in Werner syndrome [442]. Mutations in the gene coding for RECQL3 helicase produce the phenotype of Bloom syndrome. Inherited mutations in the third human homologue, *RECQL4* (8q24.3) may produce any of three phenotypes – Baller-Gerold syndrome, RAPADILINO syndrome and Rothmund-Thomson syndrome [443–445]. These three exhibit some common features, among which are congenital skeletal abnormalities (up to absent bones at birth), short stature and gastrointestinal disturbances (e.g. persistent diarrhoea). Only Rothmund-Thomson syndrome, however, resembles the premature-ageing, cancer-prone phenotypes produced by defects in *RECQL2* and *RECQL3* genes [446].

Werner and Bloom syndromes may share common features, but the respective phenotypes are different, as the affected genes produce proteins with somewhat different functions. Both conditions, however, are characterised by hypermutability, including hyperrecombinability, leading to chromosomal instability and increased risk for development of various tumours.

Werner syndrome

Werner is a typical segmental progeroid syndrome, transmitted by autosomal recessive model [447]. The clinical presentation of Werner syndrome is dominated by accelerated ageing with onset around teen age. It affects the skin (laxity, early appearance of deep wrinkles, loss of subcutaneous fat, scleroderma-like changes) as well as the internal organs (development of typical 'age-related diseases' such as cataracts, chronic obstructive lung disease, atherosclerosis, diabetes type 2, cardiovascular disease, all with unusually early age of onset). Cultured cells from patients with Werner syndrome exhibit premature replicative senescence – after about 20 population doublings, unlike normal human cells that usually stop dividing after ≈50 population doublings [448]. This was later found to be related to telomere dysfunction [449]. Somatic cells from Werner syndrome patients are prone to chromosomal aberrations – translocations, inversions, and deletions, chromosomal fusion and breakage [450]. Increased incidence of malignancy (leukemia, lymphoma, as well as solid tumours) is characteristic of Werner syndrome [451, 452]. Life expectancy with Werner syndrome rarely extends beyond 30–40 years.

The phenotype is produced by defects in the *RECQL2* gene (8p12), coding for the helicase WRN [453]. WRN helicase unwinds double-stranded DNA during repair by recombination. It

also interacts with exonuclease 1, the excision enzyme of mismatch repair, stimulating its nuclease activity [454].

Genomic instability is noted also in heterozygous carriers of Werner syndrome mutations [451].

Bloom syndrome

Bloom syndrome occurs in homozygous carriers of mutations in the gene coding for the helicase BLM (*RECQL3*, 15q26.1). Bloom syndrome is characterised by growth retardation, UV sensitivity and disorders of skin pigmentation, telangiectasias, immune deficiency and cancer-proneness [455]. Cultured cells from patients with Bloom syndrome exhibit marked chromosomal instability, specifically hyperrecombinability between sister chromatids and between homologous chromosomes [456].

The BLM helicase interacts with several proteins involved in the maintenance of genome integrity and may be redistributed and/or modified in response to genotoxic stress [457, 458]. BLM constitutes part of the BRCA1-associated genome surveillance complex (BASC), together with other DNA repair proteins, among which are ATM, MSH2, MSH6, MLH1, RFC-1, 2 and 4, and the MRN (MRE11-RAD50-NBS1) complex [459].

The cancer-proneness in Bloom syndrome is believed to be related to the increased rate of potentially pro-carcinogenic genomic rearrangements and the increased overall rate of genomic instability, which, in turn, increases the risk for loss of heterozygocity at loci containing at least one defective allele (e.g. tumour-suppressor genes) [458,460, 461].

Life expectancy in Bloom syndrome is shorter than in healthy people (mainly because of the increased cancer-proneness), but may extend beyond the 40-ties (longer then in Werner syndrome). Male patients are usually infertile, while women with Bloom syndrome may conceive naturally and carry the pregnancy to term, producing a viable newborn [462].

4.3. Human diseases and conditions associated with defects in genes coding for proteins acting in the assessment of damage, coordination and regulation of DNA repair

Monogenic or multifactorial genetic disease may be associated with carriership of allelic variants of genes, coding for proteins acting in the coordination and the regulation of DNA repair. Disease phenotypes may also arise from mutations in genes coding for proteins acting in the assessment of DNA damage and the process of decision-making about whether the cell must attempt repair of damaged DNA or reroute directly to apoptosis.

4.3.1. Li-Fraumeni syndrome and other conditions associated with carriership of different allelic variants of *TP53*

The Li-Fraumeni syndrome (LFS) results from mutations in the gene *TP53* (17p13.1), coding for the master regulator protein p53. p53 is a transcription factor, functioning in the regulation of the cell cycle, induction of programmed cell death, control of ageing, regulation of cellular metabolism, etc.

The major feature of Li-Fraumeni syndrome is cancer-proneness [463]. The lifetime risk for cancer and the risk of cancer recurrence for heterozygous p53 mutation carriers are grossly elevated – between 50 and 100 times higher than the population risk. The type and location of tumours may vary significantly and may include common types of cancers, as well as cancers which are generally rarely seen in clinical settings [464]. For reasons still not entirely clear, in carriers of germline *TP53* mutations, the penetrance of the associated disease

phenotype shows certain gender differences. More specifically, by age 50 it is 93% in female carriers and only 68% in male carriers, stabilising, however, at about 90% for both genders by the age of 70 [465, 466]. Female mutation carriers also seem to have earlier age at onset of first cancer compared to male mutation carriers. About 10% of the carriers of germline mutations of *TP53* gene (usually – females) remain asymptomatic throughout their lives.

LFS is a very rare disease, with only about 50 cases described in the specialised literature. It is transmitted in autosomal dominant pattern, that is, the carriership of one affected copy is usually sufficient to produce the characteristic disease phenotype. The reason for this is that the protein p53 carries out its functions as a homotetramer. The presence of even one altered subunit in the tetramer may seriously compromise the function of the protein (negative allelic complementation). Presumably, carriership of two defective gene copies is not compatible with life and such embryos die very early in pregnancy.

About 75% of all mutations in *TP53* gene (somatic as well as germline) are missense substitutions, resulting in decreased capacity of mutant p53 to regulate (more commonly, activate) the transcription of its target genes. When the mutations in *TP53* are inherited, the age of onset of first tumours is often directly related to the degree of loss of capacity of the mutant protein for transactivation of the target genes [467, 468]. Nearly 20% of the germline mutations affect the oligomerisation domain, while somatic mutations of p53 are usually found in the DNA-binding central domain [467].

In some patients with phenotypes, consistent with LFS, the *TP53* gene is intact and the disease-associated mutations are in the gene, coding for the checkpoint protein kinase CHK2 [469]. CHK2 acts upstream of p53, stabilising it by phosphorylation [470]. As the defects in CHK2 result in failure of the same signalling cascade, they produce a phenotype which is essentially the same as in LFS (LFS2). There is also a third locus (LFS3, 1q23) defects in which may produce a Li-Fraumeni syndrome phenotype [471].

It had been demonstrated that uterine p53 deficiency in female mice resulted in striking increase in the incidence of preterm birth, despite the fact that the experimental animals had normal ovulation, fertilisation, and implantation of the embryos [472]. A specific post-implantation mechanism was proposed, causing premature terminal differentiation and senescence-associated growth restriction of the decidual cells.

The first and foremost checkpoint in the cell cycle – the G1/S checkpoint, where the cellular DNA is inspected for potentially carcinogenic damage – is controlled by p53. Therefore, it is not surprising that *TP53* gene is a primary mutation target in cancer. Over 50% of human cancers carry mutations in the *TP53* gene, abrogating its tumour-suppressor functions [473]. In other tumours, the *TP53* gene locus or the chromosome arm containing it may be selectively lost, altogether abolishing the p53 function as a checkpoint regulator.

Several polymorphisms have been described in the *TP53* gene that do not produce disease per se and are not associated with any specific pathology [474, 475]. The capacity for induction of cell cycle arrest and/or apoptosis of these alternative forms, however, may be significantly different from the wild type allele. The Pro72Arg polymorphism is very common in all populations, with significant variation in the prevalence of the one or the other form depending on the latitude [476, 477]. The Pro47Ser polymorphism is less common [475,478, 479]. Carriership status of polymorphisms in *TP53* gene constitutes an

important part of individual repair capacity (ICR). The carriership of some of these allelic variants may play a role in the formation of the risk for various multifactorial diseases and conditions in healthy individuals. When the associated diseases and conditions have already developed, carriership of polymorphic variants may affect the outcomes and aid in the identification of possible complications. It may also affect aspects of normal healthy physiology, such as fertility, normal ageing and longevity. The alternative polymorphic variants of p53 may be differentially targeted by cancer-related mutagenesis, with the Pro allele selectively lost and the Arg allele selectively retained and mutated.

4.3.2. Ataxia-telangiectasia (A-T, Louis-Bar syndrome)

Ataxia telangiectasia is characterised by conjunctival telangiectasias, immune deficiency, ataxia and hypersensitivity to ionising radiation. The clinical presentation of A-T may be heterogeneous, with not all symptoms present in many patients and the clinical presentation varying from mild to severe.

The *ATM* (ataxia-telangiectasia mutated) gene codes for the ATM protein, responsible for induction of cell cycle arrest in response to DNA damage and DNA repair. ATM may reroute the cell's programme to apoptosis even if the p53-dependent apoptosis pathway is non-functional.

Initially, four complementation groups were proposed for A-T [480]. The linkage analysis, however, showed that they all mapped to the same locus on chromosome 11 (11q22.3), where the *ATM* gene was located [481].

A-T is transmitted in an autosomal recessive pattern, as one intact copy is sufficient to maintain a basal level of damage alertness and ATM-associated DNA repair. The prevalence of A-T is relatively higher than the prevalence of Li-Fraumeni syndrome, about 1:50,000 live births. This is probably related to the higher rate of intrauterine mortality of foetuses affected by LFS. Notably, the heterozygous carriership of ATM seems to be very common in humans, the estimates varying between 1.4% and 2.2% of the general population and even more common (up to 12.5%) in populations with marked founder effects [482–484].

Affected individuals usually present in early childhood with progressive cerebellar ataxia that may initially be misdiagnosed as cerebral palsy of the ataxic type. Later, conjunctival telangiectasias and immune disturbances emerge. Progressive neurological deterioration (without mental retardation) affects a significant proportion of older patients [485]. Thymus hypoplasia and absence or severe reductions in the levels of IgG2 and/or IgA are seen in the majority of the patients. Recurrent sinopulmonary infections (related to the accompanying immune deficiency), and gonadal dysgenesis are also common among the A-T patients. Cultured A-T cells are abnormally resistant to inhibition of DNA synthesis (induction of cell cycle arrest) by ionising radiation [486]. Individuals with A-T are hypersensitive to ionising radiation, even the normal background radiation.

Predisposition to malignancy, especially haematological cancers, is one of the hallmarks of the A-T syndrome and cancer is the most frequent cause of death in A-T patients [487]. Lymphomas in A-T patients tend to be of B-cell origin, whereas the leukemias are usually of T-cell origin.

Unlike most diseases and conditions, transmitted by autosomal recessive model, cancer-proneness related to carriership of molecular defects of *ATM* extends to heterozygous

carriers as well. A-T patients from non-consanguineous families are usually compound heterozygotes (carrying different mutations on the two gene copies). Heterozygous carriers of some of the inherited mutations in the *ATM* gene may exhibit increased propensity for various cancers (e.g. familial breast cancer, colorectal carcinomas, etc.) in the absence of typical A-T associated features.

A variant form of A-T has been described that does not produce all of the clinical signs and symptoms and/or may result in a relatively mild clinical presentation. A-T variant patients exhibit only partial deficiency of ATM, the residual levels varying between 1 and 17% of the normal level of ATM [488].

Somatic mutations in the *ATM* gene have been found in some tumours (lung carcinoma, chronic lymphocytic leukemia, T-cell prolymphocytic leukemia, colorectal carcinoma) [489–491]. The individual prognosis in colorectal cancer may be dependent on the level of expression of ATM (loss of expression or decreased expression of ATM being associated with worse prognosis) [492].

Induced attenuation of the ATM-dependent repair pathway (ATM blocking) in p53-deficient cancer cells has been explored as a therapeutic strategy in recent years. It is believed that ATM blocking may effectively cripple the repair machinery in cancer cells, rendering them incapable of repairing DNA damage caused by anticancer therapy.

4.3.3. Familial breast cancer due to mutations in the *BRCA1*/*BRCA2* genes

It has been known for quite a long time that breast and ovarian cancer could run in families, but the role of the genetic background in the constitution of the risk for development of breast and ovarian cancer was confirmed by research methods as late as the 90-ties of the XX century [493, 494].

The predisposition to familial breast/ovarian cancer is transmitted as an autosomal dominant trait, although both copies of the responsible gene must be inactivated to produce a cancer-prone phenotype. Usually, one defective copy is inherited, and the other is mutated on a somatic level, during individual life (in accordance with the 'double-hit' mechanism). The risk of development of tumours is associated with ageing (the older the individual, the higher the risk). The penetrance, however, may be very variable, depending on the type of the mutation as well as various other factors. Cases of cancer occurrence in young (below 35) carriers, and non-development of BRCA-related cancers in proven carriers until old age (70 years and more) have been recorded. The development of tumours is dependent on somatic mutations occurring during individual life and may be modified by lifestyle, occupation, and other factors (e.g. hormonal status, smoking, working with sources of ionising radiation, working late night shifts – light exposure during night hours is believed to disrupt the circadian cycle).

In 5–10% of all cases breast/ovarian cancer is familial, that is, it occurs in more than one family member (a blood relative) and in consecutive generations. The cumulative lifetime risk for development of breast cancer in the general population is estimated at about 10% (1:10). Having one first-degree relative from the direct line (either maternal or paternal) with breast and/or ovarian cancer puts the individual at twofold elevated risk for developing the same type of cancer (1:5, or 20%). Having two or more first-degree relatives

from either the maternal or the paternal line with breast and/or ovarian cancer increases the risk to five times the population risk (1:2, or 50%) [495].

The most common cause for familial breast and ovarian cancer are mutations in the tumour-suppressor genes *BRCA1* (17q21.31) and *BRCA2* (13q13.1), coding for proteins functioning in DNA repair and maintenance of genome integrity. Germline mutations account for 1–2% of all breast cancers and 15–20% of the familial cases. For carriers of mutations in the *BRCA1* or *BRCA2* genes, the cumulative lifetime risk for breast cancer is between 50 and 90%, for ovarian cancer – 40–50% [496, 497]. Risk is higher if the index patient/s in the family were diagnosed with cancer below 35 years of age. In families with history of breast cancer only, mutations in the *BRCA1* gene are found in about 15–20% of the index patients and in families with breast and ovarian cancer – 60–80%. First tumours usually appear at the age 40–60 (albeit earlier onset is not uncommon) Families with multiple occurrences of familial breast/ovarian cancer are not rare, as the affected gene if often already transmitted to the next generation before the appearance of first cancer-related manifestations.

The *BRCA1* gene is affected in the majority of BRCA1- and BRCA2-related familial cancers. The prevalence of BRCA1 mutations associated with increased risk for cancer may vary in different ethnic groups, being highest in people with Ashkenazi Jewish ancestry (about 2% of the general population, the mutation spectrum represented mainly by three or four founder mutations) [498, 499]. The prevalence of specific mutations may also vary significantly in different ethnic groups.

A third related gene, *BRCA3*, also on chromosome 13 (13q21), was shown to be responsible for a small proportion of familial breast cancer cases [500].

Mutations in the *BRCA1* gene may be associated with younger age of onset than *BRCA2* [501]. Mutations in other genes may also play a role in the constitution of the risk for development of breast and ovarian cancers (e.g. *BRCA3*, *BRCATA*, *CHK2*, etc. [502–504].

Carriership of two inherited copies of mutated BRCA1 is believed to be incompatible with life, the affected embryos dying in utero. A small subgroup of patients with Fanconi anemia (complementation group D1), however, carry either homozygous or compound heterozygous mutations in the *BRCA2* gene (see below) [505]. As carriership of mutations in *BRCA* genes is not uncommon, a small proportion of individuals at high risk may carry defects in both their *BRCA1* and in *BRCA2* genes. The associated risk is similar to the risk in carriers of a defective copy of either BRCA1 or BRCA2, albeit the first tumour/s may appear earlier and/or appear in more than one location in double heterozygotes [506, 507]. For a double heterozygote, the risk of transmission of at least one defective allele to their children is higher, about 75% (compared to the 50% risk of transmission in carriers of mutation in one of the two genes).

Mammary cancer (familial or not) may develop in males, but the risk is generally lower, as many breast tumours are estrogen-dependent. The female/male prevalence ratio in mammary cancer is about 100:1. Males with mutations in the BRCA1 or BRCA2 genes are at 6% cumulative lifetime risk of developing mammary gland cancer (about 1:16, which is more than 100 times the cumulative lifetime population risk for males).

BRCA1-associated cancers often are estrogen-independent, unlike *BRCA2*– associated cancers, which may be estrogen-dependent. In carriers of *BRCA1* and *BRCA2* mutations, cancers other than breast and ovarian tumours may develop – e.g. peritoneal cancer [508]; pancreatic cancer [509]; prostate cancer in males [510, 511] and glioblastoma [512]. The latter is not that surprising, as BRCA-1 is expressed by neural stem cells [513].

In a proportion of familial breast, ovarian and uterine cancers, the inherited defect is neither in the *BRCA1* nor *BRCA2* genes, but in the *BARD1* gene. *BARD1* gene codes for one of the subunits in a protein complex with ubiquitin ligase activity (BRCC), capable of ubiquitination of p53 in vitro [514]. The mutations disrupt the interaction between BRCA and BARD proteins [515].

Several therapeutic options are available for suspected or proven carriers of *BRCA1* and *BRCA2* mutations. One of these is watchful waiting, that is, regular examinations, (including laboratory diagnostics) for early signs of cancer. This includes more frequent (twice a year instead of once a year) breast echograms, possibly breast MRI, and screening for ovarian cancer. As soon as any sign potentially associated with cancer (even seemingly insignificant) becomes manifest, surgery and/or adjuvant therapy is undertaken so as to prevent spreading of cancer.

Use of oral contraception in clinically healthy carriers of BRCA1 and BRCA2 mutations may decrease their risk for ovarian cancer [516, 517].

In a selected group of individuals (males as well as females) at risk for familial cancer of the mammary, preventive use of tamoxifen (in women – combined with ovarian ablation) may be beneficial. Tamoxifen citrate (TC) is a classic drug typically used for treatment of advanced mammary tumours. When taken preventively, however, it may reduce the risk of breast cancer almost by a factor of two, even in carriers of mutations in the *BRCA1* and *BRCA2* genes and/or delay the age of occurrence of first tumour [518–520]. Tamoxifen belongs to the group of selective modulators of the estrogen receptor. In different tissues, the estrogen receptor may respond differently to TC, but in the mammary epithelium it acts as an antiestrogen. The affinity of tamoxifen to the estrogen receptor (ER) is relatively low. In the liver, however, it is metabolised by cytochrome P450 to its two active metabolites, 4-hydroxytamoxifen and N-desmethyl-hydroxytamoxifen, whose affinity to the estrogen receptor is about 100-fold higher [521]. The tamoxifen(metabolite)-ER complex binds to the relevant control regions in DNA in the mammary epithelial cells and inhibits the transcription of mRNA of proteins that are normally inducible by the ligand-bound estrogen receptor, including ER-responsive genes, coding for proteins acting in cellular proliferation [522, 523]. Therefore, tamoxifen does not cause death of cancer cells, but inhibits the growth of the tumour.

In most cases, tamoxifen may be taken for years without serious associated health risks. The major adverse effect of tamoxifen use is increasing the risk for endometrial carcinoma, as in the endometrial epithelium tamoxifen acts as an estrogen receptor agonist, stimulating cellular growth.

Since the risk for development of breast and/or ovarian cancer is very high in carriers of mutations in the BRCA1 and BRCA2, drastic measures such as preventative double mastectomy with or without ovarian ablation may be considered [524–526]. This virtually

eliminates the risk for breast and/or ovarian cancer tumorigenesis, which are the primary location of tumours in patients with BRCA1 and BRCA2 mutations, but it does not affect the risk for development of other tumours associated with mutations in the same genes. For tumours other than these of the breast and the ovary, watchful waiting (frequent check-ups and intervention when needed) is usually recommended so as not to miss the early signs of tumour growth.

4.3.4. Retinoblastoma

Retinoblastoma is the most common paediatric tumour– between 1:15,000 and 1:25,000 children, and about 2–3% of all tumours with age of onset in infancy or childhood [527]. It is usually diagnosed between the second and the third year of life but some of the associated signs and symptoms (strabismus, leukocoria (a late sign), inflammation, exophthalmos, etc.) may be noticed before the first year [528]. Very rarely, the onset of retinoblastoma may be in adult life.

Retinoblastoma results from inactivating mutations in the *RB1* gene, coding for retinoblastoma protein, located at 13q14.2. RB1 is a tumour-suppressor protein with a role in the induction of cell cycle arrest in the presence of DNA damage by inhibiting the progression from G1 to S phase of the cell cycle [529].

Usually, one intact copy is enough to ensure normal levels of retinoblastoma protein that would keep cell proliferation in check. Both somatic copies of the *RB1* gene must be inactivated in order to unleash tumour growth. This usually develops in accordance with the 'double-hit' mechanism. In about half of all cases, the affected children were born with one defective copy of the *RB1* gene and the second mutation, producing loss of heterozygocity, occurs during individual life. In the other 50% of retinoblastoma cases, the first 'hit' in the *RB1* gene is due to de novo mutations that has occurred during embryonic development and the second – to somatic mutagenesis [530–533]. Mitotic mutations and mitotic recombination (gene conversion during mitosis) play important roles in loss of heterozygocity in for the *RB1* gene [534].

The molecular defects in retinoblastoma may be point mutations as well as more extensive genomic rearrangements, including deletions of parts of the gene and cytogenetically significant deletions and translocations in the 13q region, producing a contiguous gene syndrome, in which retinoblastoma is one of the features [535].

The penetrance of different *RB1* mutations may greatly vary. Often enough, symptomatic children are born to asymptomatic carrier parents. About 10% of congenital carriers of one defective *RB1* copy remain asymptomatic throughout their lives [536]. Balanced translocations in asymptomatic parents involving the 13q region may become unbalanced in the offspring and produce retinoblastoma [531]. RB1 mutations may affect only the germline in clinically healthy people (germline mosaicism), resulting in affected children born to healthy non-carrier parents. The percentage of germline mutations in the *RB1* gene is between 5 and 10% in asymptomatic parents which have already had one affected child [537]. This means that the risk for them for having another affected child is only about 1:5 or 1:10 (for comparison, the risk for an asymptomatic carrier parent or a carrier, previously treated for retinoblastoma to have an affected child is about 50%). RB1 mutations arise preferentially in the male germline during meiosis [538].

In about three-fourths of all retinoblastoma cases, the tumours affect only one eye (unilateral). In the remaining 25, the tumours appear in both eyes (bilateral) or may affect the pineal gland as well (trilateral retinoblastoma). Unilateral tumours are usually products of de novo mutations (not inherited from any of the parents). Inherited mutations in one *RB1* gene copy are usually associated with bilateral or trilateral tumours, which may be more aggressive and more likely to be associated with extraocular manifestations (e.g. osteosarcoma), and may become manifest at earlier age than unilateral tumours [539]. Patients with unilateral tumours usually have better prognosis than patients with bilateral and trilateral tumours.

All individuals with retinoblastoma are at increased (over 250-fold) cumulative lifetime risk for pinealoblastoma and osteosarcoma, as mutations in the *RB1* gene play a role in the pathogenesis of these tumours as well [540].

In modern clinical settings and when diagnosed early enough, retinoblastoma is highly treatable, with 5-year survival between 85 and 90% [541, 542].

4.3.5. Autosomal-dominant adenomatous polyposis coli (familial adenomatous polyposis type 1)

Familial adenomatous polyposis type 1 (FAP1, Gardner syndrome) is related to disordered cell-cell signalling and transduction of the proliferative signals to the nucleus. It is inherited by autosomal dominant model. FAP2 produces a similar phenotype, but is transmitted in an autosomal recessive fashion and is related to mutation in a different gene (see below).

FAP1 is related to mutations in the *APC* gene (5q22.2). APC is a tumour-suppressor protein, regulating the phosphorylation and subsequent degradation of beta-catenin via the ubiquitin-proteasome mechanism [543], effectively suppressing the signal transduction via the WNT signalling pathways. The latter are more than one and may play different functions, but are strongly implicated in the stimulation of rapid division and migration of cells. The WNT pathways are often overstimulated in cancer [544, reviewed in 545]. APC/beta-catenin complexes play a role in the stabilisation of microtubules at the cell membrane, regulating the capacity for cell migration and colonisation of new sites [546–548].

The somatic copies of the *APC* gene are often deleted in cancers, particularly in colorectal carcinomas. The inactivation of APC is believed to be a crucial step in the malignant transformation of adenomatous polyps of the colon into overt carcinoma. In FAP1 one defective copy of the gene is usually inherited, and the other is inactivated on a somatic level. Individuals with familial adenomatous polyposis develop small, polyp-like tumours of the colon and the rectum, usually starting around adolescence. The number of polyps varies from several to hundreds and thousands, with risk of malignant transformation varying between 5 and 10% for every single polyp. Cancerous transformation of the polyps occurs relatively late, usually in the fourth or fifth decade of life or later.

The APC phenotype may be very heterogeneous with regard to type and location of tumours. Carriers of APC mutations may develop tumours in extracolonic locations as well – lipomas, osteomas, sebaceous cysts, thyroid cancer, carcinoma of the gallbladder, desmoid tumours, etc. [549, 550].

The general therapeutic approach in individuals with FAP is watchful waiting, with systematic check-ups for presence of polyps, removal of any growth and subsequent histological analysis to check for signs of malignant transformation. Regular examinations for cancer other than colorectal carcinoma are also highly recommended.

Multiple clinical trials have proven that use of 'classic' non-steroidal anti-inflammatory drugs (NSAIDs), specifically aspirin, may lower the risk for colorectal cancer in carriers of mutant APC alleles and even cause regression in the number and size of polyps [551, 552]. It is believed that the mechanism of suppression is related to inhibition of cyclooxygenase-2 (COX2), as it has been found to be overexpressed in many cancers (colorectal cancer, including HNPCC; bladder cancer; oesophageal cancer, and others) [553–555]. The prolonged use of specific COX-2 inhibitors has been found to be associated with decreased risk for colorectal cancer [556, 557]; but, somewhat paradoxically, with increased risk for breast cancer and haematological cancers [558].

4.4. Disorders of mismatch repair

4.4.1. Hereditary non-polypous colorectal carcinoma

In 1993, it was demonstrated that some familial colorectal cancers may be related to defects in inherited mismatch repair. Over 90% of the cases of hereditary non-polypous colorectal colon cancer (HNPCC, Lynch syndrome) are associated with defects in the human genes *MSH2* (2p21) and *MLH1* (3p22.2), homologues of bacterial MutS and MutL, respectively [295,559]. Some of the cases of familial non-polypous colorectal cancer may be associated with mutations in the *MSH6* gene (2p16.3), also a homologue of MutS [292].

Predisposition to HNPCC is transmitted by autosomal dominant model and the associated tumorigenesis follows the 'double-hit' mechanism described by Knudson [530]. Affected individuals are usually born with one functioning and one defective copy of the *MSH2*, *MLH1* or *MSH6* genes and loss of heterozygocity is usually result of a somatic mutation. The resulting hypermutability increases the risk for occurrence of other pro-carcinogenic genetic mutations and effectively, albeit indirectly, triggers the transition to cancer transformation. Carriers of mutations in the *MLH1* and *MSH2* genes are at about 75% cumulative lifetime risk of developing colorectal cancer, the risk being somewhat higher for carriers of *MSH2* mutations than for the *MLH1* mutations [560]; while for the carriers of *MSH6* mutations the risk is generally lower, between 35 and 55% [561, 562]. Males were found to be at increased risk compared to females [563, 564]. Smoking is a potent environmental risk modifier in HNPCC, with smokers being at higher risk for development of colorectal cancer, especially when combined with male gender [564].

Defects in *MLH1* and *MSH2* genes may be associated with tumours other than colorectal cancers, such as pancreatic cancer, breast cancer and prostate cancer [565, 566]; head and neck cancers [567]and precancerous states such as leukoplakia [568]. Mutations at the MSH2 and MLH1 loci may also produce Muir-Torre syndrome phenotype, characterised by a constellation of sebaceous skin tumours and cancer of various internal organs (including tumours of the gastrointestinal tract) [569].

Since HNPCC, unlike FAP, does not produce polyps (which may alert the individual about the increased cancer risk), the routine screening programmes constitute crucial part in the prevention of colorectal cancer in HNPCC. Carriers of defective copies of the genes *MLH1*,

MSH2 or *MSH6* are strongly advised to attend intensive screening for early detection of colorectal carcinoma, as well as regular check-ups for signs of cancer in extracolonic locations.

4.4.2. Familial adenomatous polyposis type 2 (FAP2)

Some of the polymorphic variants of *MUTYH* (the homologue of MutY of E. coli) in man are associated with late-onset, recessive familial adenomatosis coli (FAP2) [570]. Because of this, FAP2 is now often referred to as MYH-associated polyposis (MAP).

FAP2 is characterised by multiple adult-onset adenomatous polyps of the colon and rectum. Affected individuals are at increased risk of colorectal cancer, but may also develop endometrial carcinoma and cancers of the head and neck [571].

The human *MUTYH* gene is located at 1p34.1. It encodes an A/G-specific adenine DNA glycosylase that removes the mispaired adenine from A:G and A:oxo-G pairs. It may also remove 2-hydroxyadenine from DNA [299]. Unlike FAP1, FAP2 is transmitted in autosomal recessive manner, that is, affected individuals have inherited two defective copies of the gene [572].

Somatic mutations in the *MUTYH* gene may be seen in a small subset of patients with gastric cancer [573].

4.5. Human diseases and conditions associated with defects in base excision repair

4.5.1. It is all in the genes, just as your mother told you

Virtually all of the genetic diseases we have discussed so far fall into the category of monogenic disease (defects in one gene produce one or more distinct pathological phenotype/s). Monogenic diseases in man are usually rare (that is, their incidence is below 1:10,000 in the general population). They may exhibit different clinical severity, varying from mild (sometimes becoming manifest only in response to certain triggers – e.g. glucose-6-phosphate dehydrogenase deficiency) to severe (grossly debilitating, sometimes to a degree of being incompatible with postnatal life). Age of onset of monogenic disease may also vary significantly, some with signs and symptoms present at birth (or, with modern imaging and laboratory techniques, even diagnosable in utero), some with age of onset in childhood to adolescence (most commonly), others with adult-onset (in some cases, not manifesting until the fourth or fifth decade of life).

Monogenic diseases producing very mild, barely recognisable disease phenotypes and/or becoming manifest only in response to specific stressors may not be diagnosed as genetic disease at all. The above mentioned glucose-6-phosphate dehydrogenase deficiency usually presents as a mild condition, with haemolytic anemia developing only after ingestion of certain drugs (e.g. quinoline derivatives, sulphonamides, analgesics, acetylsalicylic acid, some antibiotics, etc.) or foods (broad beans – Vicia faba), or triggered by infections.

Another very common condition (10% in all populations), that is largely unrecognised as genetic disease is wheat gluten sensitivity (coeliac disease). It is characterised by inflammation of the mucosa of the small intestine, sometimes severe enough to result in malabsorption, malnutrition, iron deficiency and megaloblastic anemia, loss of bone density, defects of dental enamel, etc. The predisposition for sensitivity to gluten is of purely genetic origin, but whether the associated phenotype would develop depends on the grade of exposure to carbohydrate sources containing gluten (wheat, rye and barley).

Carrier individuals living in societies where the staple carbohydrate source does not contain or contains low amounts of gluten (rice, maize, etc.), the associated condition may not become symptomatic at all and the genetic disorder may be unnoticed throughout the life of the individual.

Other monogenic diseases may be misdiagnosed as something else – for example, those that occur later in life and may pass as normal 'age-related' changes, e.g.: cataract related to defects in the gene *LIM2* (19q13.421) [574]; cardiomyopathy in late life (7th decade and beyond) related to defects in the crystallin alpha-2 gene (11q23.1) [575]. Others may be confused with other late-life diseases conditions. We have already mentioned familial cancer syndromes with late onset which may produce multiple affected individuals from successive generations. These may never be identified as genetic disease unless affected individuals become symptomatic before the age of 50.

In all these cases, the disease alleles may be transmitted freely for many generations, as they do not interfere significantly with the health of the affected individuals in reproductive age and/or their reproductive fitness.

Many diseases and conditions with genetic component (even when this genetic component is crucially important, as in monogenic disease) may be unrecognised as such. For example, some severe monogenic diseases may practically never come to clinical attention – for example, those that cause early death of the affected embryos (so that affected babies are never born). Only a small part of the foetuses affected with genetic disease survive the intrauterine life. It has been estimated that only 50% of the foetuses with Down's syndrome and only 2% of the foetuses affected with holoprosencephaly live to term or near the term and are born alive [576, 577].

Sometimes, the phenotype caused by genetic disease may be misattributed to some other cause. For example, MCADD (medium-chain acyl-CoA dehydrogenase deficiency due to inherited defects in the *ACADM* gene (1p31.1)) was only identified in 1986 as the culprit in a proportion of cases of hypoglycaemia with impaired ketogenesis in infants and sudden infant death syndrome [578].

Genetic diseases may be directly associated with carriership of certain gene variants, but the resultant phenotype may be very similar to phenotypes which are produced (at least partially) by lifestyle choices. Obesity and the related condition of insulin resistance are prime examples. Certainly, both conditions may (and sometimes indeed do) result from sedentary lifestyle with very little or no physical activity outside the absolute everyday minimum (e.g. shopping, job-related activities, etc.), combined with intake of excess calories, predominantly from carbohydrates and fats. As we all know, however, this lifestyle is not always associated with development of obesity and/or insulin resistance. Obesity may develop in people with generally healthy lifestyles, if they are, for example, carriers of mutations predisposing to increased appetite and storage of large fat depots (e.g. the melanocortin-4 receptor gene, the leptin and the leptin receptor genes etc.). Similarly, insulin resistance may develop in individuals with normal weight; alpha-1-antrypsin deficiency is associated with emphysema or chronic obstructive pulmonary disease (COPD) even in never smokers, etc.

It would be prudent to say that obesity and insulin resistance are more likely to develop (and/or develop earlier) in people whose lifestyle and habits are not 'healthy', and are less likely to develop in people who exercise regularly and eat a balanced diet. Such statements, however, are not much help when it comes to assessing the risk for a particular individual to develop a particular condition. Where is the unknown factor, then, the factor that may completely compromise one's efforts to live a healthy life while allowing an unhealthy eater to fit easily in their prom outfit at the age of 50? At least partially, the answer to this question is: in their genes.

As it was repeatedly demonstrated during the last years, carrying certain allelic variants of different genes may tip the scales of likelihoods toward increased or, more rarely, ***decreased*** risk for developing the associated conditions. Carriership of 'high-risk' alleles, however, is not always a verdict, as the associated condition may not develop at all, or may be very mild and/or completely manageable. As we saw earlier, even in 'classic' monogenic diseases the penetrance of the associated diseases or conditions may not be 100% and confirmed carriers of pathological alleles may remain disease-free until very old age. Similarly, carriership of 'protective' allele variants is not an unconditional warranty for health throughout the life of the individual.

The concept of the multifactorial (partly genetic, partly environmental) pathogenesis of human disease emerged slowly in the last decades of the XX century, dispelling myths such as the myriad of 'revolutionary diet plans' that allegedly guaranteed lifetime health and fitness and facilitating the development of new ways of thinking, such as the concept of individualised medicine. The concept of multifactorialism in biology and medicine unified the apparently irreconcilable extremes of 'destiny is already written' and 'everything is subject to change', turning medical prognoses into a puzzle of likelihoods and probabilities. Confusing as this concept might be for some, at present it seems to be the best way to explain the fact why some people develop a disease and some not, despite the fact that the two groups may have the same lifestyle and the same socioeconomic status.

It was quite a surprise when it was discovered that at least some of the genes, implicated in the pathogenesis of common multifactorial diseases and conditions were actually genes coding for products acting in DNA repair and the maintenance of genomic integrity. Later, it was demonstrated that the relationship between one's genetic background with regard to capacity for repair of DNA damage, and one's phenotype may be very complicated, with the same allele/s having different weight in health and in disease, and with different consequences when viewed in different settings (e.g. in assessment of risk for developing a condition vs. assessment of eligibility for a specific therapy when the condition has already developed, and the possible post-therapeutic outcomes).

Until not very long ago, it was believed that defects in base excision repair produced such a severe phenotype that the affected embryos or foetuses died very early and never lived to term. It was only after 2000 when polymorphisms in the genes coding for proteins with a role in BER were recognised as major agents in the pathogenesis of common multifactorial diseases and conditions – cancer, metabolic syndrome, diabetes type 2, and others. Carrier status with regard to polymorphisms in BER genes may also play a role in seemingly unrelated areas such as transplantation science, as the outcomes in allogeneic

transplantations of tissues and organs may be modulated by allelic variants of genes of BER (see below).

The major genetic factors related to the capacity for repair of DNA damage by BER and their association with inherited susceptibility to certain diseases and conditions are reviewed below.

4.5.2. Polymorphisms in genes coding for proteins of BER and their association with human diseases and conditions

The initial step in repair of oxidised bases in mammalian DNA is carried out by DNA glycosylases and apurine/apyrimidine lyases that excise the altered base. In man, excision of oxidised bases is carried out by hOGG1, NTHL1, NEIL1, NEIL2 and NEIL3

Association of allelic variants of genes of BER with metabolic syndrome

Metabolic syndrome, one of the most common conditions in adulthood, associated with a myriad of adverse outcomes and having enormous impact on the quality of life of the affected individuals and their families, may actually turn out to be a disease of DNA repair (at least in a proportion of cases). Metabolic syndrome is a term used for a specific clustering of phenotypic and biochemical markers that were found to be associated with increased risk for cardiovascular disease and diabetes type 2 [579]. According to the WHO criteria, diagnostic criteria for metabolic syndrome include increased levels of fasting glucose (above 6.1mmol/L); or impaired glucose tolerance (2-hour postprandial glucose levels above 7.9mmol/L); or diagnosed diabetes (2-hour postprandial glucose levels above 11.1mmol/L); plus two or more of the following: 1) abdominal obesity (fat deposits predominantly around the midline –waist:hip ratio >0.9 in men, >0.85 in women); and/or body mass index (BMI) >30kg/m^2; 2) on antihypertensive medication or clinical hypertension (≥140mm Hg systolic/ ≥90mm Hg diastolic); plasma triglycerides ≥1.7mmol/L; HDL cholesterol <0.9mmol/L in men or <1.0mmol/L in women; urinary albumin excretion rate ≥20μg/min or albumin:creatinine ratio ≥3.4mg/mmol [580]. The prevalence of the metabolic syndrome varies between ethnic groups, but is estimated to have an average prevalence of 20–25% in adults, rising to the striking 40–45% after the age of 50; and several per cent in children (up to 50% in overweight children in the developed countries) [581, 582].

It has been proposed that polymorphic variants of the human gene coding for the apurine/apyrimidine lyases NEIL1 (15q22.33) and NEIL3 (4q34.2) may be associated with development of metabolic syndrome and diabetes type 2 [217,583]. The genotype-phenotype relationship between carriership of variants of Neil1 and metabolic syndrome was initially studied in animal models (mice and rats). In 2006, it was demonstrated that Neil1 knockout (Neil1 -/-) mice developed normally until the 4–6 months of life (which is approximately the 'mature adult' life stage in mice), then they gained weight quickly, until becoming moderately overweight (predominantly females) or obese (predominantly males) [218]. The double null mutant mice also exhibited dyslipidemia, fatty livers, hyperinsulinemia and kidney damage, and their mitochondrial DNA showed levels of damage higher than in control mice. In 2007, Roy et al. identified and characterised four rare allelic variants of human NEIL1 – Ser82Cys, Gly83Asp, Cys136Arg and Asp252Asn, two of which (Gly83Asp and Cys136Arg) exhibited virtually no enzymatic activity. The authors

speculated that individuals, heterozygous for these inactive *NEIL1* variants may be at increased risk for metabolic syndrome [584]. These variants were estimated to be rare, with prevalence below 1% in the general population, therefore, only a small part of the population affected with metabolic syndrome would carry them. A later study in patients with diabetes type 2 identified inherited mutations in the *NEIL1* gene in 2.9% of the patients and none in the control group [585]. One of the mutations was synonymous (AAA to AAG, both coding for lysine). Further research is needed in order to clarify the relationship between risk for metabolic syndrome and polymorphisms in the *NEIL1* gene.

Since the treatment would not be any different in 'hereditary' metabolic syndrome and diabetes type 2 than in 'sporadic' cases, it is currently believed that there is not much point in screening for genetic predisposition, as there are simple lifestyle measures (maintaining healthy weight, keeping arterial pressure below 140/90, correction of impaired cholesterol homeostasis, etc.) may decrease the risk for development of metabolic syndrome in patients with family history of metabolic syndrome and diabetes type 2 as well as in 'sporadic' cases.

Association with cancer

One of the *NEIL1* variants previously described by Roy et al. in 2007 was later identified in patients with primary sclerosing cholangitis with cholangiocarcinoma [584]. Another six *NEIL1* variants with reduced capacity for repair of DNA lesions were identified in patients with gastric cancer [587]. Mutations in *NEIL2* have been found, albeit rarely, in patients with colorectal cancer and squamous carcinoma of the oral cavity and the oropharynx; and lung cancer [588–591].

Association with hereditary immune deficiency syndromes

Carriership of mutations in the gene encoding human uracil DNA glycosylase (*UNG*, 12q23-q24.1) may result in immunodeficiency with hyper-IgM type 5 (HIGM5). This is, essentially, a defect in class switch recombination [592]. The condition is transmitted in autosomal recessive manner and is very rare, with only several cases reported so far. Data obtained in animal models suggests that patients with UNG deficiency may also be cancer-prone in later life, as ageing *UNG* (-/-) knockout mice have been found to develop B-cell lymphomas at a rate considerably higher than wild type controls [593].

Association with nucleotide expansion diseases

Recently, it has been proposed that trinucleotide expansions may result from irregularities in BER repair [594,595]. Expansions of trinucleotide repeats constitute the molecular basis of some genetic diseases (generally neurodegenerative diseases – Huntington's disease, Friedreich's ataxia, myotonic dystrophy, spinal and bulbar muscular atrophy, fragile X tremor/ataxia syndrome and others). The expansions usually occur during DNA replication, but may also happen during BER repair of oxidative DNA damage by a mechanism of strand slippage and formation of hairpin structures [595]. Hairpin structures generated on the daughter (newly synthesised) strand may result in expansions of the repeated unit, whereas hairpins on the template strand would usually result in deletions of repeated units [596]. What is more, it has been already demonstrated that a DNA lesion located at the 5'-end of CTG repeats would result in expansion, whereas a lesion located either in the middle or the 3'-end of the repeat would lead to a deletion [597].

It has been demonstrated in mice that the glycosylase Ogg1 plays a central role in age-dependent somatic expansion of triplets [212]. This could explain satisfactorily the phenomenon of age-related somatic expansion of trinucleotide repeats observed in murine models of Huntington's disease and myotonic dystrophy [598,599]. It occurs specifically in the brain of affected animals, where the level of oxidative stress is high as a rule and is supposedly getting higher with age. Monitoring of age-dependent somatic expansion is, of course, not possible in humans, but the mechanism is supposedly similar.

4.5.3. Polymorphisms in genes coding for products acting in chromatin remodelling and their association with multifactorial disease

Undifferentiated cells (including cancer cells) exhibit chromatin hyperplasticity, that is, the chromatin structure is significantly more relaxed and the relative amount of euchromatin is higher than that in differentiated cells. In non-cancer cells (e.g. cells of the early embryo) this is associated, on the one hand, with the high proliferative potential of the cells and, on the other hand, with the many possible differentiation routes the cell could take. In cancer cells, the predominantly relaxed conformation of chromatin facilitates DNA replication to ensure their rapid division, and altered chromatin remodelling aids in the inactivation of some genes (e.g. coding for tumour-suppressor proteins) and activation of others (e.g. coding for growth factor receptors). Disordered chromatin remodelling (by methylation of control elements, histone acetylation/deacetylation, etc.) is a characteristic feature in many cancers [600–602].

Chromatin remodelling may play a role in the constitution of the risk for other human diseases and conditions, besides cancer. The role of high-mobility group A (HMGA) proteins in the development of common multifactorial diseases other than cancer has been particularly well studied, probably because of the fact that HMGA expression is altered in cancer as well.

HMGA (formerly called HMGI/Y/C, now divided into HMGA1 and HMGA2) are non-histone proteins belonging to a family of regulatory factors with a major role in maintaining the chromatin architecture and the regulation of the expression of numerous genes. HMGA proteins are considered master regulators of gene expression, though they do not typically function by direct transcriptional activation but, rather, by altering DNA conformation [603-607]. The latter modulates the binding of transcription factors onto the AT-rich promoter/enhancer regions of their target genes. Alternatively, HMGA proteins may work by displacing histone H1, a general transcriptional repressor, from transcription initiation sites.

Disturbed regulation – usually, upregulation – of the expression of HMGA is a common finding in virtually all human cancers, resulting in ectopic expression of proteins characteristic of undifferentiated cells 608, reviewed in 609]. In this sense, the *HMGA1* gene may be considered a classical proto-oncogene. HMGA overexpression was found to inhibit BER as well as NER [610,611].

A direct relationship between the level of expression of HMGA in mitochondria and the level of accumulation of oxidative damage has been noted, associated with the risk for development of metabolic syndrome, diabetes type 2 and, possibly, cancer. Mitochondrial DNA is believed to be particularly sensitive to oxidation, due to its physical proximity to the

site where the reactive oxygen species are formed as well as the lack of protective structuring (absence of histones). Mitochondria are partially deficient in DNA repair mechanisms, that is, they do not employ the mechanism of nucleotide excision (NER) for the repair of their DNA [21]. This could be a matter of evolution-imposed parsimony, as usually the major type of DNA damage in mitochondrial DNA is oxidative, caused by the constant flow of reactive oxygen species generated by oxidative phosphorylation. The mechanisms for repair of virtually all other types of lesions may had been deemed redundant and, therefore, lost in the evolutionary process. Quantitative experiments have demonstrated the direct relationship between the level of inducible expression of HMGA in mitochondria and the level of mitochondrial dysfunction expressed as accumulation of oxidative damage [21].

In 2005 Foti et al. described a hyperglycaemic/obese phenotype in mice deficient in the Hmga1 protein and hypothesised that a similar mechanism operated in human metabolic syndrome and diabetes type 2. An unusually high level of oxidative damage was found in the DNA of the Hmga (-/-) cells. The same group reported that in some patients with severe, early-onset type II diabetes the expression of HMGA1, a major modulator of the chromatin structure, was markedly reduced [612,613]. Increased levels of oxidative stress have been identified as one of the hallmarks of insulin-resistant diabetes and atherosclerosis for decades now [614], but whether it was the DNA damage that induced and promoted the insulin resistance or was it the latter that induced high levels of DNA damage, it was not clear.

Over 20 allelic variants of the human *HMGA1* gene have been identified so far, with at least 4 of them found in heterozygous state in individuals with severe insulin resistance [615,616]. These *HMGA1* variants affected the coding portions of the gene as well as the untranslated region, indicating that the preservation of the wild type sequence and the regulation of the expression of the gene were equally important. The two studies have not identified homozygous carriers of any of these 'high-risk' variants. This may mean that carriership of two defective copies may dramatically decrease the fitness of the carrier individuals, causing early death or incapacitation at early age. The studies in HMGA (-/-) knockout mice support this, as development of cardiac hypertrophy starting from the age of 2 months was reported in nearly all the adult homozygous animals [613]. Taken together with the high level of oxidative DNA damage seen in insulin resistant cells, a hypothesis has been recently proposed that faulty management of DNA damage may play an important role in the etiopathogenesis of insulin resistance and atherosclerosis [617,618], but the exact induction mechanism still remains elusive.

It could be hypothesised that inherently low levels of HMGA (e.g. resulting from polymorphisms in the respective gene/s) may, via different mechanisms, decrease the efficiency of protection of the cell's DNA against oxidative damage. This may result in accumulation of oxidative lesions, some of which may have carcinogenic potential (as mutagenesis is essentially a random process). Once the cell is transformed, it may deploy the cancer-specific mechanism of increasing the level of genome instability, resulting in constitutive overexpression of altered, tumour-specific forms of HMGA, stimulating further the malignant growth. In this light, insulin resistance and metabolic syndrome could be

viewed as a long-term, low-grade premalignant state in which DNA damage is slowly accumulating, until the repair machinery of the cell fails to resist the constant oxidative attack and surrenders to neoplastic transformation [619]. This, however, takes a long time and usually occurs in late adulthood and in old age, while the primary conditions of insulin resistance and diabetes type 2 may cause fatal complications much earlier. Maintaining the fine balance between normal and pathological states becomes even finer and more difficult to manage as the organism ages. Since the prevalence of both cancer and insulin resistance increase with age, it is hard to differentiate the impact of the one or the other, but it could reflect a chain of probabilities and events that are causally linked, but the determining factors are too strictly regulated at so many levels in non-transformed cells that the resulting effects would only show with advancement of the ageing process.

4.6. Other inherited diseases and conditions related to defects in DNA repair genes

4.6.1. Predisposition to melanoma

But in case you should happen to get in any salve that gives
people **more freckles**, feel free to send me seven or eight jars.
Astrid Lindgren, Pippi Longstocking (1945).
Translated by F. Lamborn

More than 10 different genes have been identified mutations in which may be associated with increased risk of melanoma. Among these are genes, associated with repair of DNA and/or cell cycle arrest in response to damage, such as *XRCC3*, *CDKN2A* and *CDK4* [620-622], as well as several others, such as the gene coding for matrix metalloproteinase 8 [623] and genes coding for receptor tyrosine kinases, such as *ERBB4* [624]. The *CMM1* locus (1p36) is known to be associated with significantly increased risk for melanoma, but the gene function is still unknown [625]. Functional polymorphisms in the genes coding for two of the major matrix metalloproteinases (MMP2 and MMP3) were not found to confer increased risk for melanoma development or to be associated with melanoma progression [626-628]. Mutations in the *BRCA1* gene are also believed to be associated with increased risk for melanoma [629]. Carriership of some of the common polymorphisms in the XPC gene is associated with predisposition for melanoma and other cancers because of decreased capacity for recognition of DNA damage in the non-transcribed regions of the genome [630]. Families with hereditary melanoma are often at increased risk for developing colorectal carcinoma as well [631].

It is worth noting that primary melanomas may actually be pigment-free, which implies that it may actually be missed on dermatological examinations. Sometimes, the diagnosis of melanoma could only be made after a biopsy in other parts of the body shows metastatic lesions typical of melanoma.

There is one single independent modifying genetic factor for the risk of skin cancer, however, that must always be taken into consideration as it works independently of genetic predisposition for skin cancer. Namely, the amount of the UV-protective pigment melanin in the skin may affect the risk for melanoma as well as non-melanoma cancer, regardless of the presence of predisposing genetic factors such as the polymorphisms mentioned above or environmental factors (UV exposure). Differently pigmented skin contains different

amounts of melanocytes and thus the ability to withstand UV damage may considerably vary between skin types.

For the purposes of assessment of proneness to sunburn and pigmentation after trauma to the skin as well as cancer risk, the most commonly used Fitzpatrick scale differentiates six skin types [632,633].

Table 3. Fitzpatrick scale of skin tone with regard to proneness to sunburn and relative cancer risk [632,633].

Fitzpatrick skin type	Characteristics	UV Sensitivity	Risk of UV-induced skin cancer
I	Very fair and/or freckled skin, red or light blonde hair	Highly sensitive, always burns, never tans	High
II	Fair skin and fair hair, e.g. fairer Caucasians; also some Asians with fair skin	Very sun sensitive, burns easily, tans minimally	High
III	Light to medium brown skin, e.g. darker Caucasians, some Asians with darker skin	Sun sensitive skin, sometimes burns, slowly tans to light brown.	Moderate
IV	Olive or medium brown skin, e.g. dark Caucasians (Mediterranean type), some Hispanics	Minimally sun sensitive, burns minimally, always tans to moderate brown.	Moderate
V	Brown or dark brown skin, e.g. darker Hispanics, some Blacks	Sun insensitive skin, rarely burns, tans well.	Lower than skin types I–IV
VI	Dark brown to black Blacks	Sun insensitive, never burns	Lower than skin types I–IV

The skin type most sensitive to UV is Fitzpatrick type I (Table 3). It is very fair and prone to freckling, and practically never pigments in response to skin trauma. Type I skin is usually seen in individuals with red or light blonde hair and blue or green eyes, but may occasionally be seen in people with darker hair colour and/or dark eyes (Fig. 13).

a)b)c)

Figure 13.

a) Classic Fitzpatrick type I skin, hair and eye colour and more unusual combinations of pale skin;

b) Fitzpatrick type I and dark hair and green eyes;

c) skin type I with dark hair and dark eyes.

Images: American Girl. These images are included under the fair use exemption and are restricted from further use.

Currently, freckling is considered to indicate for a very sensitive skin with a history at least one serious sunburn, likely to have occurred in childhood. It is also believed to be an alert for diminished DNA repair capacity in the skin, calling for regular use of sunscreen throughout the year (not only the spring and summer months) and regular check-ups by a dermatologist.

The least UV-sensitive Fitzpatrick skin type is VI, which is a deep shade of black, never burns and may pigment heavily even after minor trauma. Having deeply pigmented skin, however, is no guarantee that unprotected exposure to sunlight or other UV sources would not eventually trigger the development of skin cancer. Indeed, dark-skinned, dark-haired individuals may also develop UV-induced cancer (indeed, not as often as pale-skinned people).

It is common for the patients with familial predisposition for melanoma to have fair complexion, often with freckles, blue or green eyes and numerous nevi (moles) on the face and the body [634,635]. The moles are usually somewhat larger than average size, with irregular borders, pink to brown in colour. This is believed to reflect the primary defect in the distribution and the properties on the melanocytes. Neither skin colour nor genetic susceptibility for melanoma, however, matter very much, when taken on their own. It is only when the endogenous factors (fair skin colour and carriership of polymorphisms conferring increased risk for skin cancer) occur in an individual living in environment rich in exogenous factors (frequent sunburns, possibly occurring at early age), the difference in the risk for skin cancer may become significant. Thus, a fair-skinned, repair-deficient individual is at higher risk for development of UV-associated skin cancer than a dark-skinned, repair-proficient individual, even if the former had fewer occasions of sunburn than the latter.

While virtually all eukaryotic organisms need UV light in small doses, UV-associated carcinogenesis in man is a serious problem worldwide. According to data from World Health organisation (WHO), the incidence of skin cancer (non-melanoma and melanoma alike) is steadily increasing in the last few decades, affecting 0.2–0.5% of the general population (that is, between 1:500 and 1:200 people). Until relatively recently, the long-wavelength component of UV spectrum (commonly known as UV-A) was considered to be almost harmless with regard to DNA damage, and, respectively, skin cancer risk. Several years ago it was shown that UV-A may cause thymine dimers in DNA of skin fibroblasts [636]. Indeed, the duration of exposure to cause sufficient DNA damage was found to be longer with UV-A than with UV-B. Also, it was shown that UV-irradiation, regardless of the wavelength, increases the systemic risk for carcinogenesis by a mechanism of lowering (albeit temporarily) the immune defences of the organism [637-639].

Prolonged UV irradiation may cause or stimulate the development of premalignant skin changes such as dysplastic skin moles and actinic keratoses. A thorough dermatological check-up is advised if any of these appears de novo anywhere on the body or is perceived to be changing in some way (e.g. size or colour), regardless of history of sunburn.

UV-irradiation (including sunburn) may induce temporary immune system suppression (local as well as systemic) [640,641].

4.6.2. Fanconi anemia

Fanconi anemia (FA) may result from defects in any of more than 10 genes coding for proteins functioning in DNA repair, usually in direct or indirect association with the BRCA1 and BRCA2 proteins [642,643]. Mutations in different genes are associated with different complementation groups of FA. FANCA, FANCC, FANCE, FANCF, FANCG and FANCL proteins form a nuclear complex that activates a related protein (FANCD2) during the S phase of the cell cycle or following DNA damage (crosslinking agents) [644,645]. Activated FANCD2 co-localises with the breast cancer susceptibility protein, BRCA1 in damage-induced foci and in synaptonemal complexes of meiotic chromosomes [645, 646]. The *FANCJ* gene codes for a helicase, functioning in repair by recombination [130]. One complementation group of Fanconi anemia, D1, is related to double (homozygous or compound heterozygous) mutations in the *BRCA2* gene [505].

Clinical features of Fanconi anemia include developmental abnormalities, growth retardation, early-onset myelodysplastic syndromes and cancer-proneness (especially leukemia, but also solid tumours). Cultured cells from patients with FA exhibit high rate of chromosomal aberrations [647]. Patients rarely live beyond 30–40 years.

5. Repair proteins with signalling and effector functions

The time to repair the roof is when the sun is shining.

J. F. Kennedy (1917–1963)

Nature has invented a system of mechanisms ensuring the flexible balance when it comes to decisions on whether to repair an error in DNA in order to restore the initial structure; or let it be for the sake of changeability; or, eventually, whether to sacrifice the cell altogether. This system is capable of assessing and integrating the signals from the damaged genome and of delivering the final decision about the destiny of the cell. The type, the severity and the scope of DNA damage as well as the current status of the cell and – probably – of the organism are all taken into account in order to determine the mode of behaviour after an activating event has occurred. The system for assessment of DNA damage has many direct players, uses many signalling pathways and is capable of a complete revision of the cellular programme. Some of the crucial agents are presented below; others are mentioned throughout the book.

5.1. Checkpoints and checkpoint controls in the cell cycle

Here life has death for neighbour.

Algernon Charles Swinburne, The Garden of Proserpine (1866)

Cell proliferation is regulated by a complex set of rules and restrictions, with the sole purpose of not letting cells whose DNA has been damaged divide further. The integrity of the cellular DNA is checked and verified at more than one points during the progression into the cell cycle and the type and the quantity of genotoxic damage is differentially assessed, so as not to produce mutated cell progeny. This comes at the expense of many cells dying because of failure to comply with the requirements of a certain phase in the cell

cycle. Basically, one phase of the cell cycle must be completed before entering the following phase. Failure to prepare for the next cell cycle phase may render the cell unable to progress further until the requirements of the current phase are fulfilled (e.g. DNA damage is repaired) or may initiate the concerted cascade of events ultimately leading to cell death by apoptosis.

In the presence of unrepaired DNA damage, eukaryotic cells deploy one of three major pathways: cell cycle arrest that allows time for DNA repair; apoptosis that quickly and, figuratively speaking, bloodlessly eliminates cells with irreparable DNA damage; or, in special cases, when replication of the damaged region is unavoidable, cells may resort to alternative mechanisms such as replicating through the damaged region (Fig. 14). The latter is a temporary resort and carried out at risk of introduction of mutations, as translesion DNA polymerases can handle damaged templates, but the risk of misincorporation of nucleotides is much higher than with 'routine' DNA polymerases [648].

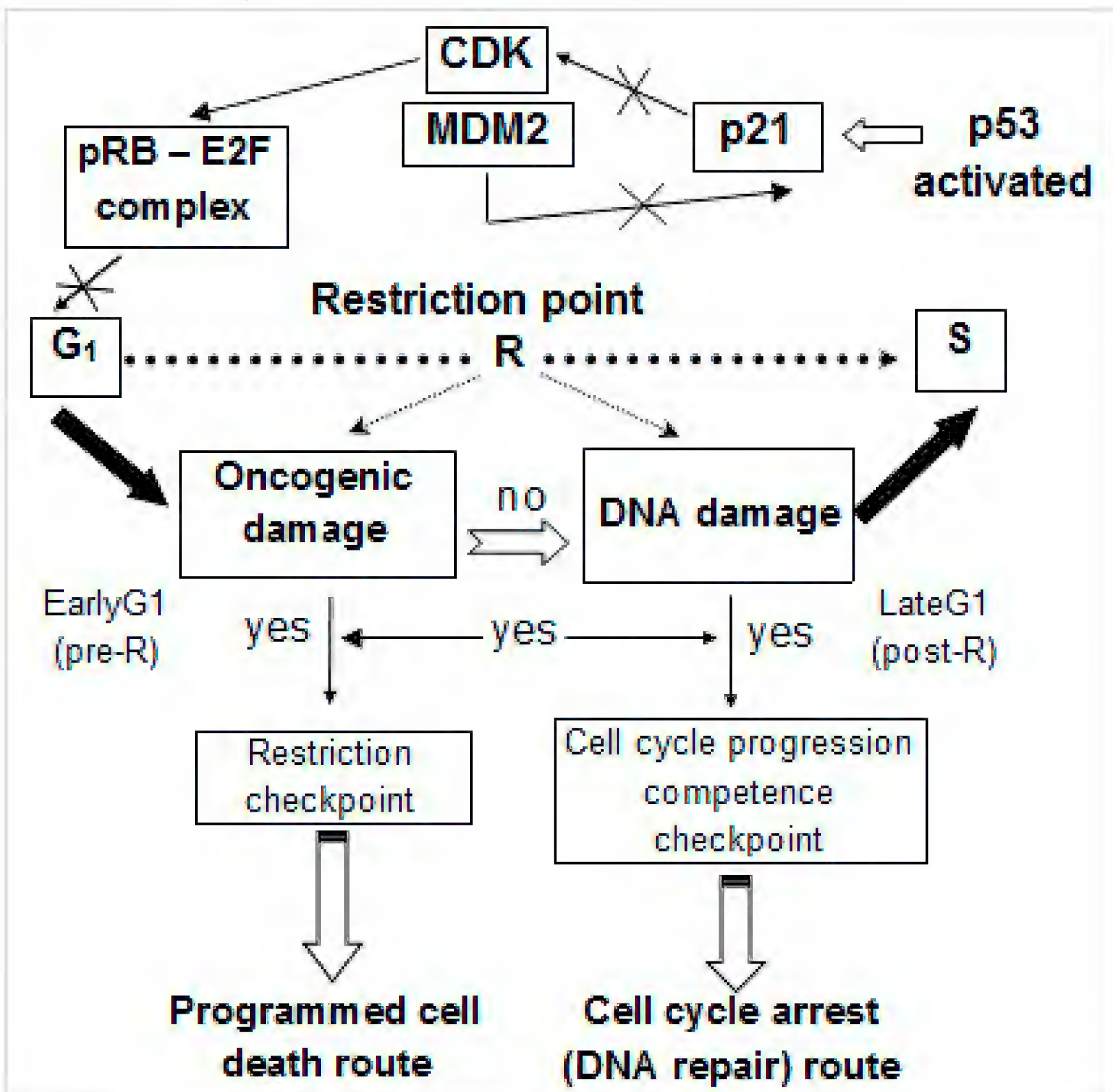

Figure 14. Major checkpoints in the cell cycle. pRB – retinoblastoma protein (see below); E2F- transcription factor E2F, part of the pRB-E2F repression complex, CDK – cyclin-dependent kinase, P21 (Cip1, WAF1) – CDK-interacting protein 1, a CDK inhibitor (see below).

The topological and temporal sites where the mechanisms of surveillance over genomic integrity operate in order to ensure that the cell would proceed smoothly through mitosis

are often referred to as checkpoint controls. Dividing cells have to be able to pass at least three crucially important checkpoints during different phases of the cell cycle – namely: G1/S phase checkpoint (often subdivided further into a restriction checkpoint (checks for presence of oncogenic damage) and a competence checkpoint (checks for DNA damage)), intra-S-phase checkpoint (Fig. 14), and the G2 phase and M phase checkpoints, each corresponding to phase of the cell cycle [649-651].

Not all cell cycle checkpoints are dependent on the presence of DNA damage and not all checkpoints are irreversible [650,652]. Usually, it is the pre-replicative G1/S checkpoints where the cells may permanently halt the progression into the cell cycle. This could happen for many different reasons. Some of these are accidental (e.g. presence of unrepaired DNA damage; deficit of nucleotide precursors, etc.), and after they have been corrected or restored to the initial state, the cell cycle may continue as usual. Another reason may be the need for the cell to permanently exit cell cycling because of terminal differentiation or impending senescence. When the need to halt the progression through the cell cycle occurs after the cell is past the G1/S checkpoints, the S phase may be postponed by slowing down the progression of replication forks and delaying the activation of the late replication initiation sites. In general, the intra-S-phase checkpoint is independent of the presence of DNA damage; rather, it serves to ensure that the replication has been completed before the physical division of the cell would begin [652,653].

The G2/M checkpoints allow for repair of DNA that had suffered damage in late S or in G2 phase of cell cycle, but before mitosis. The G2 checkpoint (also called topoisomerase II checkpoint) is also considered relatively independent on the presence of DNA damage, as it makes sure that all knots and tangles in DNA (resulting from replication) had been resolved before proceeding to mitosis [654].

The M phase checkpoint is actually comprised of no less than three separate sub-checkpoints, associated with microtubule formation and assembly of the mitotic spindle (spindle assembly checkpoint); the correct division of sister chromatids between daughter cells, and a mitotic exit checkpoint [655-657]. It has been demonstrated that the G2 checkpoint, the spindle assembly checkpoint and the mitotic exit checkpoint may act synergistically with earlier checkpoints in conditions of replicative stress, reversing the cell cycle to earlier mitotic phases [650,658,659]. As the post-G1 checkpoints cannot operate independently, however, the G1 checkpoints are the essential elements of the mechanism that would not allow cells with damaged DNA to divide.

5.2. p53 (TP53), the guardian angel and the archangel of the eukaryotic genome

These things I warmly wish for you:
Someone to love, some work to do,
A bit o' sun, a bit o' cheer,
And a guardian angel always near.
Irish blessing

The protein p53 (transformation-related protein 53 (TP53), according to the HUGO gene nomenclature committee) is a transcription factor, tumour-suppressor and master regulator of the cell cycle. p53functions in the maintenance of the integrity of the genome by transactivation (rarely, by trans-inhibition) of other genes, resulting in induction of cell cycle

arrest and DNA repair; translesion transactions; altering the cell's metabolism and/or the expression pattern of various proteins; and/or programmed cell death.

Ever since its discovery, p53 has been attributed many various properties (sometimes contradictory to each other), and it has risen to the challenge magnificently. Several thousands of p53 binding sites have been identified across the human genome, with over 120 direct targets for binding of p53 [660]. It has been estimated that the proportion of human genes regulated by p53 directly or indirectly is between 2 and 4% [661,662]. p53 was chosen as Molecule of the Year for 1993 [663]. It has been so famous for such a long time (in a research area as dynamic as molecular biology, thirty years is long time), that it is rather surprising that a Nobel prize had never been awarded for its discovery. The latter may be the reason that references to p53 have not penetrated yet into the media and the popular culture (as was the case, for example, with disorders of excision repair of DNA and telomere length).

The discovery of p53 has been reported in the same year (1979) simultaneously by four research groups, using different approaches [664-667]. It is rather curious that the era of p53 started with the protein being mislabelled as a positive regulator of cell proliferation, that is, a proto-oncogene. This stemmed mainly from the findings that p53 was predominantly localised in the nucleus; that the level of p53 mRNA and the rate of the synthesis of the relevant protein increased markedly before the S phase of the cell cycle; and that the levels of p53 were several orders higher in transformed human cell lines than in homologous nontransformed cells [668]. The fact that cell lines could be immortalised by cDNA clones encoding p53 [668-670] strengthened the notion that it was a master cellular proto-oncogene. This was the official opinion up to the 90-ties of the XX century, when it was established that cancer-specific p53 may be quite different from the wild type protein [671,672].

Not only was the function of p53 incorrectly identified at first, but its very name is, in fact, inaccurate. 'p53' is a trivial (albeit very popular) name, usually attributed to the molecular weight of the protein (allegedly 53 kDa). This is not correct, as p53 isoform 1, which is considered to be the canonical sequence, has molecular weight of only 43.653 kDa. According to UniProt (Cellular tumour antigen p53, Homo sapiens, retrieved 23 Dec 2013), in non-cancerous cells exist at least 8 other isoforms, resulting from internal promoter usage and alternative splicing [673-675]. The molecular weight of any of these alternative isoforms is lower than the molecular weight of isoform 1 and nowhere near 53 kDa. The error in determining the molecular weight was related to the specific electrophoretic pattern in denaturing polyacrylamide gels of products of cell-free translation of mRNA of eukaryotic cells treated with DNA damaging agents. Under denaturing conditions, p53 would migrate in the gel at a rate close to the rate of the 58 kDa molecular standard, probably because of its high proline content [676].

Human p53 protein is encoded by the *TP53* gene, situated on the short arm of chromosome 17 (17p13.1) [677]. The gene spans 20 Kb of genomic DNA and comprises 11 exons, the first exon having regulatory rather than coding functions. Three promoters have been identified in theTP53 gene, located, respectively, the first 100 to 250 bp upstream of the first exon, the second within the very long (≈10 Kb) first intron, and the third in intron 4 [673,678].

p53 binds to DNA as a homotetramer. Each subunit in the tetramer contains one protein molecule, which can be divided into six (or, according to some authors, seven) domains, two of which have DNA-binding properties (Fig. 15) [679,680]. The N-terminal (transactivation) domain binds damaged DNA non-specifically, while the central domain binds DNA both specifically and non-specifically.

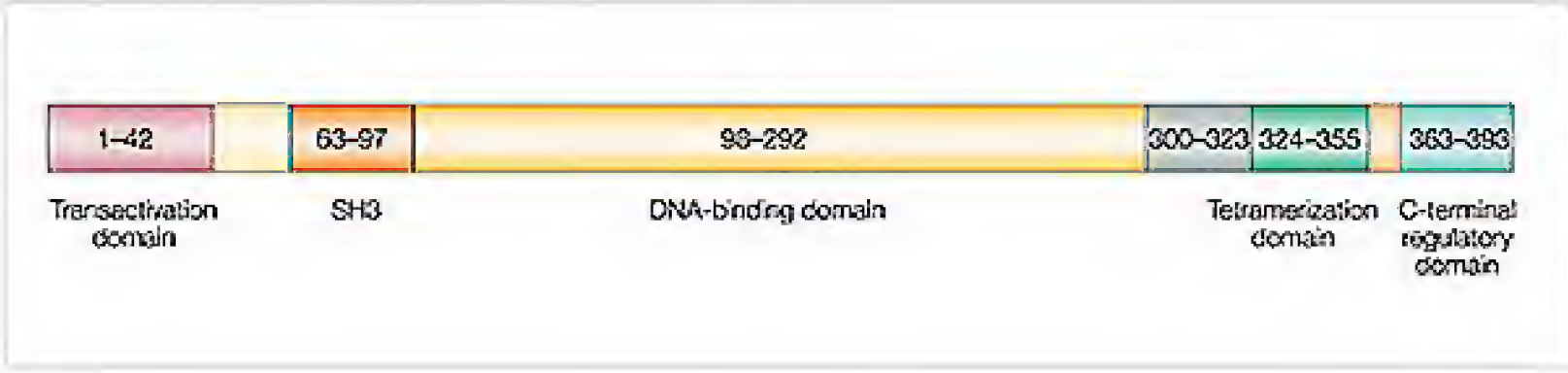

Figure 15. Domains in p53 protein [from: 681].

Specific binding of 53 via the DNA-binding domain occurs on consensus sequences consisting of two half-site copies of the 10 bp motif 5'-PuPuPuC(A/T)-(T/A)GPyPyPy-3', (Pu-purine, Py-pyrimidine), separated by 0–13 bp linker sequence [682,683]. The half-site motif consists of two quarter-sites, with one subunit of the p53 tetramer binding to one quarter-site. A single copy of the half-site motif is not acceptable for effective binding of p53 to its target genes, and even subtle alterations of the motif usually result in loss of affinity for p53.

Presence of even one defective subunit of p53 (due to inherited or somatic mutations in the *TP53* gene) may decrease or altogether abolish the DNA-binding and transcription-modulating properties of the whole tetramer (negative allelic complementation). Heterozygous carriership of one defective copy of the *TP53* gene is associated with the development of the very rare inherited condition Li-Fraumeni syndrome (LFS), characterised by increased cancer-proneness [463]. Most disease-associated *TP53* mutations, however, are of somatic origin. As a major negative regulator of cell cycle and inductor of cell death in response to damage, *TP53* is one of the primary targets of cancer-related gene inactivation. Somatic defects abrogating the function of p53 or enabling bypass of the p53-dependent control of cell cycle are found in more than half of human tumours [684,685]. The presence of inactivating mutations or loss of heterozygocity in the *TP53* locus is a predictor of poor prognosis in cancer, as it is generally associated with high tumour aggressiveness [686-689]. The typical target of p53-abolishing mutations in human cancers is the central DNA-binding domain, while germline mutations (causing Li-Fraumeni syndrome) usually affect the oligomerisation domain [467]. In normal cells under genotoxic or other type of stress, p53 is stabilised and retained in the nucleus, so that it could launch the expression of different genes involved in cell cycle arrest in order to repair the damage or, if repair is impossible, reroute the cell to the apoptotic pathway. The oligomerisation domain of p53 contains two putative nuclear export signals (NES), therefore, it is hypothesised that proper oligomerisation of the four subunits masks the NES, retaining the protein in the nucleus, while mutant variants of p53 would be promptly exported to the cytoplasm [690-692]. In non-stressed normal cells, virtually all isoforms of p53 except for isoform 9 are predominantly nuclear in G1 and largely cytoplasmic during S and G2 [673]. Predominant

cytoplasmic location of p53 in tumours (colorectal carcinoma, breast carcinoma) is usually associated with poor prognosis for the patient [693,694].

When bound to DNA, the p53 protein acts in different cis/trans interactions with its target sequences, modulating the transcription of various target genes [695,696]. Relatively recently it was found that the p53 molecule may actively slide along the length of the DNA molecule, searching for its cognate control elements [697,698].

p53 is capable of activating both the initiation and the elongation from promoters for RNA polymerase II, as it can bind directly to TFIIH [699,700]. In non-transformed cells p53 can repress the transcription from RNA polymerase I and III [701,702]. Some oncoproteins, such as the E6 oncoprotein of HPV can induce derepression of this transcriptional control [703,704]. p53 may repress the transcription from RNA polymerase I in an alternative manner – by preventing the formation of a functional transcription complex onto RNA polymerase I-specific promoters [705].

p53 is capable of inducing apoptosis not only via modulation of transcription of target genes, but by non-transcriptional mechanisms as well. In 1995 it was observed that overexpression of a mutant p53, lacking most of its DNA-binding domain and deficient in its transactivation functions, could nonetheless induce apoptosis [706]. Later, it was shown that drugs that could induce mutant p53 protein with aberrant conformation to adopt wild type conformation could also induce BAX-dependent apoptosis even in absence of transcription [707,708]. Under conditions of genotoxic stress, a fraction of p53 translocates to the outer mitochondrial membrane and increases its permeability by forming an inhibitory complex with the anti-apoptotic proteins of the Bcl-2 family. The result is release of cytochrome c, a powerful trigger of the endogenous apoptotic pathway [709,710].

p53 has been known to be capable of inducing autophagy as well as inhibiting it, which may be accomplished in transcription-dependent as well as in transcription-independent manner [reviewed in 711].

The regulation of p53 is subject to various negative or positive feedback loops. In non-stressed cells p53 is fairly unstable, maintained on but a basal level via ubiquitination by the ubiquitin ligase MDM2 712, reviewed in 713]. Ubiquitination of p53 promotes its degradation and serves as an inhibitor to its transactivation activities. p53 positively regulates the transcription of MDM2, enabling a negative feedback loop to avoid premature replicative arrest and/or death in non-stressed cells [714]. In the presence of DNA damage or other type of cellular stress, MDM2 is degraded by ubiquitin-specific protease [715], allowing the stabilisation and activation of p53 [473,716]. The *MDM2* locus and/or the related *MDM4* gene (see below) may be amplified in tumours, resulting in suppression of the pro-apoptotic properties of p53 [reviewed in 717]. MDM2 is phosphorylated at several sites (predominantly on serine residues, but also on tyrosines and threonines) by ATM upon DNA damage, usually resulting in inhibition of p53 degradation. For example, phosphorylation of MDM2 at Ser395 decreases the capacity of MDM2 to export p53 from the nucleus, resulting in its accumulation in the nucleus [718]. Phosphorylation of MDM2 at other sites, however, (e.g. Ser166) may stimulate MDM2 targeting to the nucleus, stimulating the ubiquitination of p53 [reviewed in 719]. Another crucial regulator of p53 is the Mdm-2-like p53-binding protein, Mdm4 (MDM4 or MDMX in humans) [720]. MDM4

binds to p53 and suppresses p53-mediated cell cycle arrest and apoptosis. It also inhibits the degradation of MDM2 721, reviewed in 722]. Phosphorylation of MDM4 induces the activation of p53 [723].

p53 is the major decision-maker about whether to repair the DNA, tolerate DNA damage (e.g. by the mechanism of translesion synthesis) or, in case nothing else works, kill the cell. This is tightly associated with the levels of p53 in the cell, as the mechanisms that regulate p53 levels usually operate in a dose-dependent manner. p53 binds to different types of p53 response elements with variable affinity with the length of the spacer sequence between the half-sites possibly being related to p53-DNA binding affinity [reviewed in 683]. Cell cycle arrest-related genes and some of the pro-apoptotic genes with shorter spacers in their p53-binding site bind to p53 with high affinity [724,725]. Longer spacers in the p53-binding site of the gene (e.g. in PIG3 gene) are usually associated with low binding affinity [680,726]. This allows staging of the p53-associated response, allowing for the high-affinity targets to be activated predominantly under conditions in which a strong response must be mounted quickly. Some of the p53 isoforms are also capable of modulating p53 activity, especially cancer-specific isoforms [673,674].

Activation and deactivation of p53 may be achieved by various post-translational modifications, among which are the already mentioned ubiquitination and also phosphorylation, acetylation, methylation, SUMOylation, etc. [681,690]. Acetylation is usually an activating mechanism for p53, increasing its affinity for DNA, and is normally related to induction of cell cycle arrest and/or apoptosis. It has been shown that p53 acetylation destabilises the p53-MDM2 interaction and enables the p53-mediated stress response [727]. Methylation of the promoter of *TP53* is a tumour-specific mechanism of inactivating the expression of p53, albeit it is more of a supplementary than a primary route [728,729].

Phosphorylation of p53 normally results in decreasing the p53's affinity towards MDM2, activating p53 [730]. Another major player in decision-making about whether the cell should continue in the cell cycle or not – the ATM kinase – acts upstream of p53, phosphorylating it upon DNA damage, thus activating the p53-related pathways of cell cycle arrest and/or apoptosis. p53 may be induced in an ATM-independent manner as well, provided that the damage is severe and/or extensive enough [716,731,732]. This requires the presence of various factors, among which prominent is the protein kinase CHK2 [470,733]. CHK2 (see below) acts upstream of p53, phosphorylating it on a serine residue on position 20 in the polypeptide chain. This results in increased p53 stabilisation, eventually producing cell cycle arrest in G1 in response to DNA damage [470,734]. ATM is also capable of rerouting the cell to programmed cell death in a p53-independent manner (see below).

p53 may be activated by the sole presence of damage in DNA, but it is also activated when the cell is deficient in precursors of any of the nucleoside triphosphates used in DNA synthesis. This is the working principle of some commonly used anticancer agent, such as 5-fluorouracil (blocks the biosynthesis of nucleoside triphosphates). p53 is also activated in cases when DNA or RNA polymerases are stalled for reason other than presence of DNA

damage (e.g. in the presence of alpha-amanitin, and some antibiotics such as actinomycin D, novobiocin, and others) [735,736].

If after the damage assessment the cellular DNA is deemed irreparably damaged, p53 would typically 'switch on' the programmed cell death routine of the damaged cell, thus acknowledging the needs of the many (the tissue, the organ and the organism) more important than the needs of the few (the damaged cell/s). The pro-apoptotic properties of p53 are usually deployed by inducing or, less commonly, repressing the transcription of its target genes [737]. Some types of cancer cells may abrogate the transcriptional control of p53 or they may terminate wild type p53 altogether in order to turn the p53-dependent suicide pathway off. The latter may be carried out in the simplest possible manner, e.g. via induction of aneuploidy (e.g. losing the short arm of chromosome 17 or deleting the genomic region in the vicinity of the *TP53* locus); or by methylation of the *TP53* promoter; or by making wild type p53 mRNA and/or protein unavailable or unstable. For example, the E6 oncoprotein from human papillomavirus (HPV) types associated with high risk for cervical carcinoma (16, 18) binds to a cellular ubiquitin ligase (E6-AP), thus inducing the degradation of p53 via the ubiquitin-dependent proteolytic pathway, abolishing the opportunity to dispose of infected cells by apoptosis [738]. E6 from HPV types associated with low risk for carcinogenic transformation of the affected cells also binds to E6-AP, but with significantly lower affinity, allowing at least part of p53 to escape degradation and launch programmed cell death [739].

Not until long ago, the result of any modification (germline or somatic) to the wild type sequence of *TP53* was considered to be, almost invariably, cancer. It is now known that there are subtle polymorphic variants of *TP53* that do not alter significantly its DNA-binding properties, but may cause differences in the capacity of the resultant protein to induce transcription of its target genes and/or apoptosis. Among these, prominent is the pro72-to-arg (Pro72Arg) polymorphism in the *TP53* gene [476]. It is very common in all populations. The two alternative variants of the Pro72Arg polymorphism are conformationally indistinguishable and exhibit comparable DNA-binding affinities. The p53 (Pro) variant, however, is a stronger inducer of transcription than p53(Arg), whereas p53(Arg) induced apoptosis more effectively and is more potent suppressor of transformation than p53(Pro) [474]. The carriership of both polymorphic variants of p53 may be advantageous or disadvantageous to the individual, depending on many other factors, of endogenous as well as of exogenous origin. For example, it was shown that homozygous carriers of the Pro72 allele in patients with predisposition to hereditary non-polypous colon cancer had earlier age of onset and a poorer prognosis than the carriers of at least one Arg allele and especially than the Arg/Arg homozygous genotype [740]. Carriership of Arg/Arg genotype, however, is not always a good predictor in cancer, as studies of HPV-related cervical cancer show prevalence of Arg/Arg carriers in patients with overt cancer but not in patients with cervical intraepithelial neoplasia (CIN) [741]. The age of the individual may also play a role.

5.3. ATM

ATM (ataxia telangiectasia-mutated) protein is a sensor and signalling protein with serine/threonine kinase activity. ATM-mediated pathways play a crucial role in the cellular response against double-strand breaks in DNA (agents such as ionising radiation,

radiomimetic drugs, etc.) [742,743]. ATM detects DNA breaks, induces temporary cell cycle arrest in any of the G1, S or G2/M checkpoints, activates the machinery for DNA repair and primes the apoptotic pathways [reviewed in 744 and 745]. It regulates a wide variety of downstream signalling proteins and provides an important functional link between genome integrity and the progression in the cell cycle. ATM is involved in DNA damage repair, DNA replication and recombination, cell cycle checkpoint control and homologous recombination during meiosis [746-748].

ATM functions upstream of p53, activating it by phosphorylation in response to DNA damage, but is capable of triggering the apoptotic pathways independently of p53 as well [749-751]. ATM deficient cells are unable to detect levels of DNA damage which usually result in p53-dependent activation of the DNA repair machinery [752,753]. They are similarly incapable of inducing cell cycle arrest in the presence of DNA damage, resulting in unrestricted replication of damaged DNA and multiplication of errors. Unrepaired DNA damage continues to accumulate until a certain critical level beyond which the cell is directed to a suicide route by an alternative, p53-independent mechanism [716,753]. The characteristic phenotype of ataxia/immune deficiency seen in A-T patients is due to very high levels of death by apoptosis in selected cell populations – specifically, immunocompetent cells in the thymus (hence the immune deficiency) and neurons the cerebellum (hence the ataxia). During the formation of the T-cell receptor repertoire in the thymus, a significant amount of double-strand DNA breaks occurs naturally. After the physiological recombination in the maturing immune cells is complete, however, the breaks must be ligated to restore genomic integrity. The neurons in the cerebellum and in the brain as a whole are subjected to massive amounts of oxidative DNA damage. Moreover, neurons tend to inhibit selectively the genome-wide DNA repair, focusing on the repair of actively transcribed genes. In the absence of a mechanism for sensing the damage early enough, in both cell types damage would rapidly reach levels that would trigger activation of the apoptotic pathway.

The *ATM* gene belongs to a class of housekeeping genes, coding for proteins phosphorylating key substrates in various signalling pathways, called phosphatidylinositol-3-kinases, PI3K [754]. The ATM locus spans about 150 Kb of genomic DNA, and has 66 exons [755]. ATM shares a bidirectional promoter with another housekeeping gene (NPAT) located around 0.55 Kb upstream of the ATM start codon [756-758]. The estimated promoter activity on the ATM side is about 3 times higher than on the NPAT side. ATM mRNA is subject to extensive alternative splicing, affecting the 5'- untranslated region of the gene (specifically, the first 4 exons) [759].

ATM is expressed as a 350 kDa nuclear protein comprised of 3056 amino acids [760,761]. Unactivated ATM is kept in homodimeric or higher order multimeric form. In response to DNA damage (double-strand breaks are the best studied trigger), the dimeric or multimeric ATM complexes undergo rapid autophosphorylation, resulting in their dissociation and release of active ATM monomers [762]. ATM interacts with a number of downstream substrates, among which are p53 and BRCA1 and BRCA2 proteins, as well as many others with control functions in the cell cycle.

ATM causes the stabilisation and activation of p53 partly by phosphorylating it at a selected serine residue (Ser15) [749]. ATM also phosphorylates the major negative regulator of p53, the ubiquitin ligase MDM2, decreasing the capacity of MDM2 to export p53 from the nucleus [718,763]. The activated p53 transactivates a series of downstream proteins, eventually resulting in G1-to-S transition arrest (Fig. 14).

ATM is part of the multi-subunit complex BASC (BRCA1-associated genome surveillance complex), containing tumour-suppressor proteins, DNA damage sensor proteins and signal transducers [459]. In response to DNA damage, ATM may phosphorylate BRCA directly at selected serine residues [764,765] or indirectly (phosphorylating CHK2, which, in turn, phosphorylates BRCA1) [766] (Fig. 16).

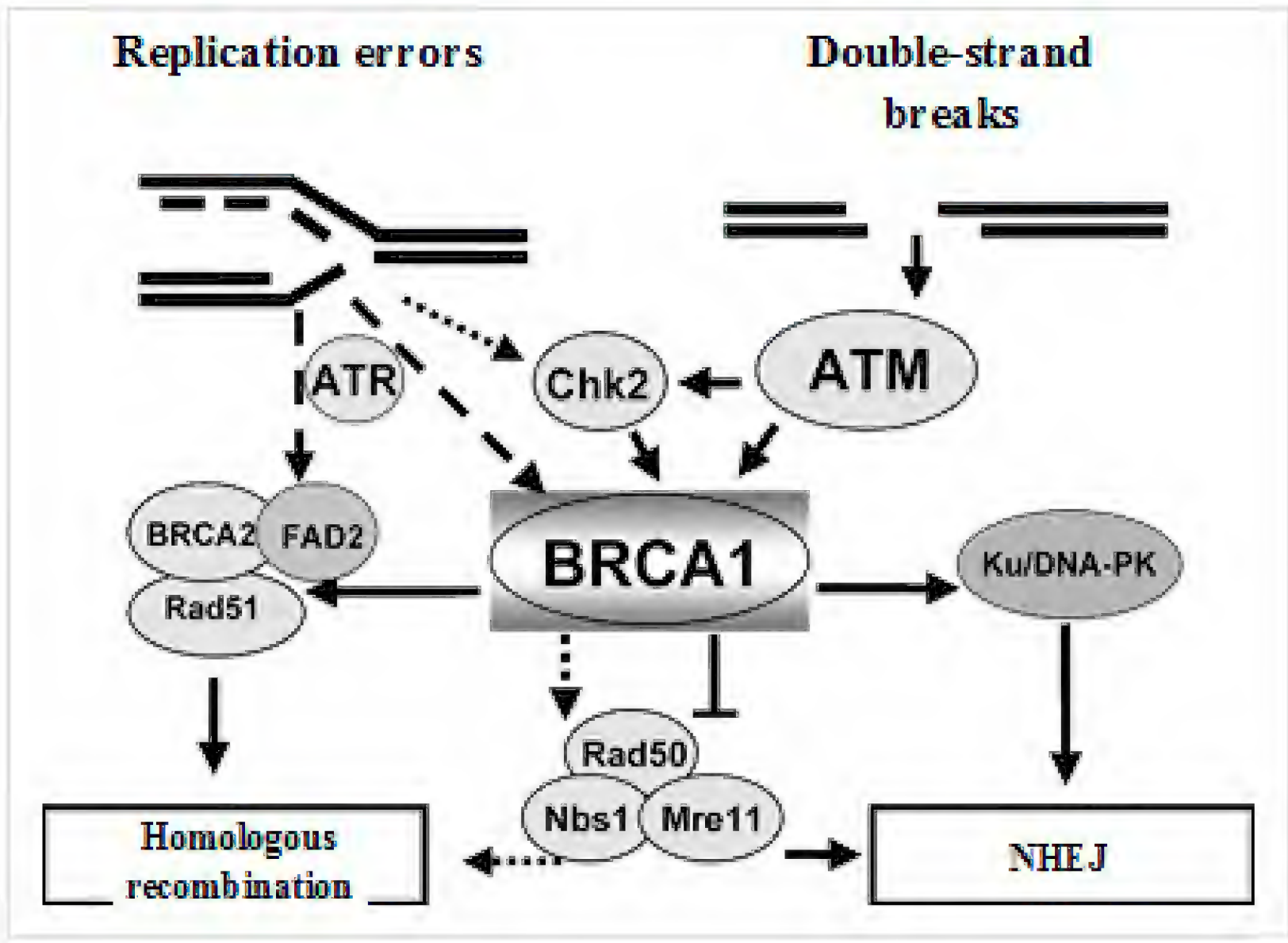

Figure 16. Different mechanisms of activation of BRCA1-controlled pathways in response of DNA damage.

ATM may induce p53-independent cell cycle arrest after the G1/S phase checkpoint, in G2/M phase. The G2/M checkpoint is associated with checkpoint kinases CHK1 and CHK2. CHK proteins phosphorylate the phosphatase CDC25C required for the removal of the inhibiting phosphate residue from CDK1/cyclin B complex (see below). The phosphorylation results in inactivation of CDC25C, thereby preventing the entry into M phase [734,767].

ATM-dependent activation of the G2/M checkpoint may also be implemented via the variant histone H2AX [768]. H2AX is one of the three variants of the core histone H2A. It contains a conserved serine-containing motif at the carboxy-terminus that is target for phosphorylation in the presence of double-strand breaks [769]. H2AX is normally buried within the chromatin microenvironment, but upon damage it becomes exposed due to local topological changes mediated by RAD51, and is rapidly phosphorylated at a specific serine residue (in mammals, this is Ser139) [150,770]. H2AX is one of the earliest of the downstream target substrates of ATM, and other members of the phosphatidylinositol-3-

kinase-like family, such as ATR and DNA-dependent protein kinase [771,772]. Phosphorylated H2AX (γH2AX) acts as a docking site for variety of other proteins involved in DNA damage response pathways, including the already mentioned MRN complex (MRE11, NBS1 and RAD50) [151,763]. These initial events activate, in turn, more of the pre-existing inactive ATM molecules, effectively multiplying the signal. Activation of ATM also involves acetylation at selected lysine residues by lysine acetyltransferase 5 (KAT5, Tip60) [774]. ATM and KAT5 act as a complex, activated by DNA damage [775].

Unlike p53, which is constantly degraded under normal conditions and only allowed to stabilise in the presence of a triggering event, such as DNA damage, ATM protein levels do not typically rise and fall in response to DNA damage. Neither does change the localisation of ATM throughout the cell cycle [776,777]. Under certain conditions, however, the pattern of expression of ATM may be altered, which is accompanied by a corresponding change in ATM activity [778-780]. ATM may be subject to transcriptional regulation at promoter as well as at mRNA level [781-783].

ATM is one of the major targets of novel anticancer therapies. The ATM-regulated damage response pathway is independent of p53-associated pathways. Tumour cells often abolish the p53-dependent mechanisms of activation of DNA repair, as p53 transactivates the pro-apoptotic pathways as well. In such cells, the only checkpoint that may remain functional is the ATM-controlled G2/M checkpoint where the cells may still induce cell cycle arrest to repair the DNA damage, producing resistance to anticancer agents. Inhibition of the ATM-controlled repair in cancer cells may increase their sensitivity to genotoxic treatments [784]. The surrounding normal cells would presumably be less affected by treatment with ATM inhibitors, provided that they have retained wild type p53 and are, therefore, still capable of G1/S cell cycle arrest. Compounds with ATM-inhibiting action, such as KU-55933, KU59403, VE-821, Torin and others are currently being developed and tested for the purposes of sensitising cancer cells to genotoxic damage of iatrogenic origin [783,785-788]. Dose-control trials intended to destroy cancer cells while affecting healthy cells only minimally are currently under way.

5.4. ATR

ATR (ataxia-telangiectasia and RAD3-related protein) is another checkpoint kinase, similar to ATM. It is a member of the phosphatidylinositol 3-kinase-related (PI3K) family of proteins, involved in cell cycle progression, DNA recombination, and detection of DNA damage [789,790]. The human *ATR* gene is located at 3q23.

ATR and ATM participate jointly in an early event in sensing of DNA damage, namely, phosphorylating of the RAD17 protein [791]. The latter is a chromatin-bound protein, similar to replication factor C (RFC). ATR protein is activated by DNA breaks (e.g. caused by UV or IR irradiation), or simply by the presence of single-stranded DNA (ssDNA) – for example at stalled replication forks or generated as an intermediate in DNA repair. ATR, in complex with ATR-interacting protein (ATRIP) is recruited to the damage sites by the presence of ssDNA bound to the single-strand binding protein RPA [792]. ATR phosphorylates and activates many proteins that are directly involved in induction of cell cycle arrest in the presence of DNA damage and DNA repair, such as BRCA1, RAD17, RPA2,

SMC1, p53 and checkpoint kinase 1 (CHK1, see below) [793-797]. ATR phosphorylates histone variant H2AX in the vicinity of sites of DNA damage [798] and in meiosis [799].

ATR deficiency in cultured cells results in genome instability and appearance of chromosome fragile sites under conditions of replicative stress [800]. Fragile sites are hotspots for chromosomal rearrangements (translocations, deletions) and sister chromatid exchanges [801,802]. Inherited ATR deficiency in man is associated with a phenotype of telangiectasias, patchy alopecia, hair and nail abnormalities and cancer-proneness (transmitted in an autosomal dominant manner) or as Seckel syndrome-1 (autosomal recessive), characterised by growth retardation, mental retardation and microcephaly with a characteristic 'birdlike' facies [803,804].

5.5. Cyclins, CDKs, and CDK inhibitors

When DNA damage is present, the progression in the cell cycle is typically halted in order to prevent production of progeny carrying altered DNA. The control over the cell cycle is executed at multiple levels and may be temporarily delayed or altogether aborted. The latter may precede apoptosis or may trigger replicative senescence. Many different types of molecules and supramolecular complexes act in the control of progression in the cell cycle, ensuring that a cell would only replicate its DNA and implement physical division if all checkpoint requirements are fulfilled (all nucleotides needed for DNA synthesis available, no signs of potential carcinogenic alteration in DNA, no signs of persistent DNA damage). Most of the control molecules of the cell cycle function as negative regulators, that is, their presence or accumulation above a baseline level would normally trigger signalling cascades that would eventually cause cell cycle arrest. Some are positive regulators, that is, their presence or accumulation beyond a certain threshold constitutes a positive signal, stimulating the cell to carry on with the cell cycle. Some of these regulators are expressed at a steady level while the levels of others would rise and fall (oscillate) during the different phases of the cell cycle and/or depending on whether the cell proceeds to division uneventfully or had sustained damage.

Cyclins and the associated cyclin-dependent kinases (CDK) are a major group of proteins functioning in the regulation of the progression through the cell cycle. Cyclins share a common conserved sequence motif – the cyclin box – which facilitates the association of cyclins with their specific CDKs and regulate the kinase activity of the resulting complex [805-807].

The different phases of the cell cycle are characterised by variation in the levels of expression of different types of cyclins. Typically, the level of the relevant cyclin would rise during a discrete cell cycle phase (e.g. in S phase – cyclin A, in G2 phase – cyclin B, etc.), or in the transition between phases (e.g. cyclin E – between G1 and S phase), then would rapidly fall. An exception is, for example, cyclin D, the levels of which level gradually rise throughout G1 phase to reach a peak in S phase, the gradually fall throughout G2 phase to reach a minimum in M phase. Some cyclins (for example, cyclin B) are regulated at a post-translation level, e.g. by proteolytic degradation in order to decrease their levels, while others (e.g. cyclin C, cyclin E) are subject to transcription control, so it is the level of expression of their mRNA subject to control rather than the level of protein [808-810].

The family of cyclin-dependent kinases (CDK) comprise >10 proteins with kinase activity involved in the regulation of the cell cycle. CDKs are typically expressed at a steady baseline level, but in order to be activated they must be associated with the respective cyclin (Table 4). Thus, the activities of cyclin-dependent kinases oscillate during the different phases of the cell cycle while their protein levels remain relatively constant.

Table 4. Basic types of cyclins, their respective CDKs, and putative functions of the cyclin/CDK complex [754,811-821]. Other types of cyclins (e.g. I, J, etc., also exist).

Cyclin	CDK pairing partner	Functions
A	CDK2	S phase, G2 phase
B	CDK1	M phase
C	CDK3, CDK8	G1 phase, transcription
D	CDK4, CDK6	G1 phase
E	CDK2	G1 to S transition
H	CDK7	CDK-activating kinase, transcription
p35 (CDK5R1)	CDK5	Transcription
T	CDK9	Transcription
L	CDK11	Transcription, pre-mRNA splicing
F	None (orphan cyclin)	Centrosome homeostasis Fidelity of mitosis
G	None (orphan cyclin)	DNA damage-induced cell cycle arrest in G2/M
O	CDK2	Apoptosis in selected cell types
X(Y)	CDK14 CDK16	Regulation of cell division in spermatogenesis Regulation of transcription of specific proteins

Most cyclins have two forms, carrying the same letter index but different number index, e.g. cyclin A1 and A2, B1 and B2, L1 and L2, etc. The two isoforms of the same cyclin are usually similar in structure and functions but may have different pairing partners (that is, besides their respective CDK kinase, which is usually the same for both isoforms).

Some cyclin-like proteins were initially classed as cyclins because of the presence of the cyclin box motif, but do not actually function in the regulation of the cell cycle at all, or play but a small role in the regulation of the proliferation in selected cell types. Cyclins T, L, F and G, as well as p35, the pairing partner of CDK5, only have the cyclin box motif in common with the 'typical' cyclins, although both groups of proteins are more or less related. For example, cyclin T is related to cyclin C [814] and has a CDK pairing partner (CDK9), but does not play a role in control of cell proliferation [822]. Similarly, cyclin F, the largest (in terms of molecular mass) human cyclin is related to cyclins A and B [811].

Most cyclin-like proteins function in transcription, interacting with the RNA polymerase II complex, but they may play other roles as well, including indirect regulation of cell proliferation. For example, cyclin T1/CDK9 complex (sometimes called positive transcription elongation factor B, P-TEFb) phosphorylates the carboxy-terminal domain of the large subunit of RNA polymerase II, thereby facilitating transcription elongation [822]. Cyclin L1 and L2 regulate RNA polymerase II and pre-mRNA splicing [823]. Cyclin X(Y) plays a role in the regulation of the cell cycle by modulating the expression of proteins implicated in the control of the cell cycle (including proto-oncogenes, e.g. c-MYC) [818].

Cyclin F is an 'orphan' cyclin, as it has no CDK partner [824]. It plays a role in M phase, ensuring the fidelity of mitosis and specifically the fidelity of centrosome duplication. Cyclin F is the substrate recognition component of an E3 ubiquitin-protein ligase complex that mediates the ubiquitination and subsequent degradation of CP110, a protein implicated in centrosome duplication. The ubiquitination of CP110 during G2 phase prevents accidental reduplication of the centrosome [819].

Cyclin G is p53-inducible and plays a role in the G2/M arrest after DNA damage [813,815].

Cyclin O is a cyclin A-like nuclear protein that reaches peak levels during G1 phase, then the levels gradually decline throughout S and G2 phases. Cyclin O was originally thought to have DNA uracil glycosylase activity, but was later classed into the cyclin family [811,825]. Cyclin O has been found to play a role in the induction of apoptosis in selected cell types (e.g. the lymphoid line) [826].

For some of the proteins that were originally believed to be 'orphan' cyclins, a partner CDK was identified later. For example, cyclin X (also called cyclin Y) is a pairing partner of CDK14 and CDK16 and plays a crucial role in spermatogenesis [817,820].

CDK5 has a pairing partner – p35 (neuronal CDK5 activator, CDK5R1) protein, which is not a cyclin, albeit it exhibits weak similarity to cyclins [827]. The CDK5/p35 system functions in modulation of transcription. In some tissues, CDK5 is known to induce stabilisation and activation of p53, promoting programmed cell death [828]. p35 is typically expressed at its highest levels in terminally differentiated (usually, non-dividing) cells, such as CNS neurons. A truncated form of p35 – p25 has been found to accumulate in brains of patients with Alzheimer's disease [829]. This is accompanied by constitutive activation of CDK. Mouse knockout models lacking p35 exhibit gross cortical layering abnormalities and severe neurological deficits [830].

The binding of cyclin to its respective CDK activates the kinase activity of the latter. The activated complex cyclin-CDK phosphorylates and activates other downstream proteins, typically related to cell division. Phosphorylation by CDK may also inactivate other proteins, e.g. the tumour-suppressor protein pRB1. This results in induction of a pro-proliferative signalling cascade by causing release of the pRB1-bound E2F, allowing it to activate its downstream genes (for details, see below) [831,832].

CDKs are usually activated by removal of a crucially important inhibitory phosphate residue from the active site of the enzyme. The phosphatase activity is carried by a family of specific highly conserved phosphatases (CDC (cell division cycle) 25 phosphatases) [833]. Three isoforms of CDC25 phosphatases were identified in man, with CDC25A acting predominantly in the G1-S transition and CDC25B and CDC25C functioning at G2/M checkpoints [834]. Phosphorylation of the CDK1/cyclin B complex (often called M-CDK) activates CDC25, promoting the progression to the M phase. Genes of the CDC25 family are classed as proto-oncogenes, as their activity is often deregulated in cancer cells [834,835]. Various CDC25 inhibitors are currently under study as anticancer agents, but none has reached clinical application yet.

The CDK-cyclin complexes containing CDK1, 2, 4 and 6 are usually activated by phosphorylation on a threonine residue by the cyclin-activating kinase (CAK) [754,836]. CAK

is made of three subunits – CDK7, cyclin H and the assembly factor MAT1 [837,838]. CAK makes up the kinase domain of the transcription factor TFIIH [839].

Cyclins and CDKs may play a role in programmed cell death. For example, the activity of the cyclin A – CDK2 complex may be up-regulated in apoptotic cells [838,839].

Regulation of CDK-cyclin levels is generally at post-translational level. The repression of CDK activity is implemented by specific CDK-inhibitors. A prominent member of the family of CDK inhibitor proteins is Cip1 (cyclin-dependent kinase inhibitor 1A, CDKNA1, p21/WAF) [840,841]. Human p21 is coded by the CDKN1 gene (6p21.2). Its expression is p53-inducible and the upregulation results in cell cycle arrest [842]. p21 represses the activity of CDK2 and CDK4, complexed with cyclin A, D and E, thus regulating the transition through the G1 to S phase of the cell cycle [843-845].

CDKN1B (p27, KiP) is another cyclin-dependent kinase inhibitor, similar to p21, which may induce cell cycle arrest in response to DNA damage or differentiation signals. p21 also plays a role in apoptosis [846,847]. CDKN1B inhibits the phosphorylation of histone H1 and pRB1 by cyclin-CDK2 and cyclin-CDK4 complexes with cyclin A, B1, D2 and E [848]. As a result, the cell cycle is blocked in G0/G1 phase. In animal models carrying targeted deletions of both *Cdkn1b* copies, terminally differentiated cells (which are normally incapable of division) re-entered the cell cycle, divided, then died by apoptosis [849].

The *CDKN2A* gene (9p21.3) utilises alternative reading frames to code for 2 major proteins: p16 (INK4), also known as multiple tumour suppressor 1 (MTS1), functioning as cyclin-dependent kinase inhibitor; and p14 (ARF), which binds to the p53-stabilising protein MDM2 [850]. Thus, the protein product of the *CDKN2A* gene controls both the p53-regulated and the pRB1-regulated pathways [851,852]. p16 modulates the activity of CDK4 and CDK6, whereas p14 acts, albeit indirectly, to prevent the activation of the CDK2/B1 complex [853].

The *CDKN1C* gene (11p15.4) encodes p57 (KIP2), a negative regulator of cell division, inhibiting several cyclin/CDK complexes in G1 [854]. The gene is affected by genomic imprinting, with the maternal allele being preferentially expressed [855]. Inherited defects in the maternal copy of the gene in man are associated with IMAGE syndrome (intrauterine growth retardation, metaphyseal dysplasia, adrenal hypoplasia congenita, and genital anomalies) [856].

Synthetic CDK inhibitors (CDK-specific or pan-inhibitors) are currently under trials as adjuvants in anticancer therapy. Among these are R-roscovitine (seliciclib), flavopiridol, difluoromethylornitine, aminopyrimidines (ZK 304709 and derivatives), and others [857-860].Some CDK inhibitors exhibit selectivity towards specific CDKs. For example, R-roscovitine is more selective towards CDK1, CDK2, CDK7, and CDK9 than towards CDK4, CDK6, and CDK8 [861]. Pan-inhibitors of CDK activity (multitargeted CDK inhibitors) work on all types of CDKs. Used in therapy together with DNA damaging agents, CDK inhibitors may aid in sensitisation of cancer cells to genotoxic therapies. The application of CDK9 inhibitors in the treatment of cardiac hypertrophy and infections with virus strains resistant to antiviral agents (e.g. drug-resistant HIV) is also intensively studied at present [861-863].

5.6. Checkpoint kinases

Checkpoint kinases (CHK, CHEK) are serine/threonine kinases acting in checkpoint-associated cell cycle arrest and activation of DNA repair in the presence of DNA damage or stalled replication forks. The *CHEK1* gene is locates at 11q24.2, the *CHEK2* gene – at 22q12.1.

CHK2 and CHK1 are downstream targets of ATM or ATR, respectively. The CHK proteins are activated by DNA double-strand breaks (CHK2) and single-stranded DNA (CHK1) [reviewed in 784]. Checkpoint kinases phosphorylate the phosphatases CDC25A, CDC25B and CDC25C; resulting in their inactivation, export from the nucleus and/or degradation [734,864,865]. Inactivated CDC phosphatases are prevented from removing the inhibitory phosphate residue from CDK-cyclin complexes, inducing checkpoint-dependent cell cycle arrest [866].

CHK1 may also be activated in response to DNA damage by the so-called "9-1-1 complex". This is the trivial name of a trimeric eukaryotic DNA clamp complex, made of the Rad9, Hus1, and Rad1 proteins [867]. It is capable of activating damage-associated signalling independently of the ATM/ATR [868,869]. In response to genotoxic treatments, 9-1-1 complex inactivates cyclin-dependent kinases and activates CHK1, suppressing replication of damaged templates [870].

Catalytically active CHK2 produces arrest in G1 phase of the cell cycle in response to DNA damage [470]. CHK2 was found to co-localise with BRCA1 in the nucleus and to phosphorylate BRCA1 at a specific serine residue (Ser988) upon DNA damage [871]. CHK1 is believed to be responsible for the S and G2 checkpoints, as CHK1-deficient cells exhibit defects in S and G2 phases of the cell cycle [866]. CHK1-depleted tumour cells are typically arrested in G2/M phase and may be subsequently rerouted to the programmed cell death pathway [872].

Several CHK1 inhibitors (UCN-01, CHIR-124, CBP-501, etc.), are currently in clinical trials as potentiating agents in chemotherapy, acting to increase the sensitivity of cancer cells to DNA-damaging agents (e.g. gemcitabine) [873-875].

5.7. BRCA1 and BRCA2

Human *BRCA1* (17q21.31)and BRCA2 (13q12.3) are housekeeping genes coding for DNA-binding proteins with central roles in the response to DNA damage [494,876]. BRCA1 and BRCA2 proteins usually coexist in the same protein complex and co-localise at damage-induced nuclear foci and the developing synaptonemal complexes in meiotic chromosomes [646]. The two proteins are subunits in a larger enzyme complex with ubiquitin ligase activity (BRCC, containing also RAD51, BARD1 and other proteins) [514]. BARD1 (BRCA1-associated RING domain-1) is a regulator of BRCA1-dependent apoptosis [877,878]. BRCA1 exhibits structural homology to BARD1, especially with regard to the DNA-binding motif (the RING domain), as BRCA1 contains zinc finger motif of the C3HC4 type. The BRCC complex is rapidly recruited in response to stalled replication forks and at sites of DNA damage [514,879] The heterodimer BRCA1/BARD1 also functions in the assembly of the mitotic spindle [880].

One of the primary downstream targets of BRCA protein complex is GADD45 (growth arrest and DNA damage-inducible gene-45), a nuclear protein, expressed predominantly in non-dividing (replicatively quiescent) cells. GADD45 inhibits the entry into S phase of the cell cycle and stimulates DNA repair at the G2-M checkpoint [881,882].

Except as a complex, BRCA1 and BRCA2 proteins may also have functions of their own. BRCA1 is a component of a large multi-subunit complex containing tumour-suppressor proteins, DNA damage sensing proteins and signal transducers titled BRCA1-associated genome surveillance complex (BASC) [459]. Among the other BASC proteins with functions in DNA repair are, for example, the protein kinase ATM, the helicase BLM, the proteins of mismatch repair MSH2, MSH6, MLH1, and the RAD50-MRE11-NBS1 complex. Also, BRCA1 is involved in the regulation of transcription of genes that are inducible upon occurrence of DNA damage, eventually producing damage-associated cell cycle arrest [883]. BRCA1 has been shown to inhibit the proliferative signalling mediated via estrogen-receptor alpha [884].

BRCA2 functions in repair of double-strand breaks by homologous recombination but not in repair by NHEJ [885].

Eukaryotic O6-methylguanine DNA methyltransferase may bind to BRCA2 and induce its degradation [886]. Targeted degradation of BRCA2 has been explored as an option for increasing the sensitivity of cancer cells to alkylating agents [887].

5.8. Poly-(ADP-ribose)-polymerase 1

Poly-(ADP-ribose)-polymerases (PARP) are members of a protein family comprising about 15 proteins in mammals. A unifying trait is the specific structure of the catalytic site (the PARP signature) – a block of 50 conserved amino acid residues forming a complicated structure made of a beta-sheet, an alpha-helix, a 3_{10}-helix, a beta-sheet, and an alpha-helix [888]. The functions of many proteins of the PARP family are still unknown, but besides their functions in DNA damage-related signalling, they seem to play roles in the regulation of membrane structures, and the maintenance of the actin cytoskeleton. Different PARP proteins may have different intracellular localisation. Many of them are cytoplasmic, others are located predominantly in the nucleus, and others may shuttle between the cytoplasm and the nucleus in different phases of the cell cycle [889].

PARP1 is the best studied member of the poly(ADP-ribose)-polymerase family in man. The human *PARP1* gene is located at 1q42.12. It encodes a nuclear protein that localises to sites of DNA damage [890]. PARP1 protein (EC 2.4.2.30) is a primary damage sensor molecule activated by DNA breaks (occurring due to the action of damaging agents as well as DNA breaks resulting from repair-related activities) [891,892]. PARP1 catalyses the transfer of multiple ADP-ribose units from NAD+ to various proteins, including the PARP1 molecule (automodification) [893,894]. The modified proteins carry long branched polymers made of APD-ribose, which serves as a triggering signal for recruitment of DNA repair machinery and chromatin remodelling. PARP1 action is associated with activation of the p53-dependent pathways of damage response [895]. Many of the targets of PARP1 are proteins playing a role in the maintenance of chromatin structure and its remodelling in response to various triggers – histones from the nucleosomal core and histone H1, HMG proteins, and topoisomerases I and II, to name only a few [896-898]. The poly-ADP-ribosylation of the target proteins of PARP1 decreases their affinity for DNA (by amassing negative charge), causing relaxation of the DNA packing at the damage site and facilitating the access of the repair machinery [899]. The initial signal generated by the presence of damage is effectively amplified by the action of PARP1, as it modifies multiple target molecules. PARP1 is,

therefore, believed to be a signalling molecule as well, acting to advertise the presence of DNA damage to the cellular repair machinery (Fig. 17).

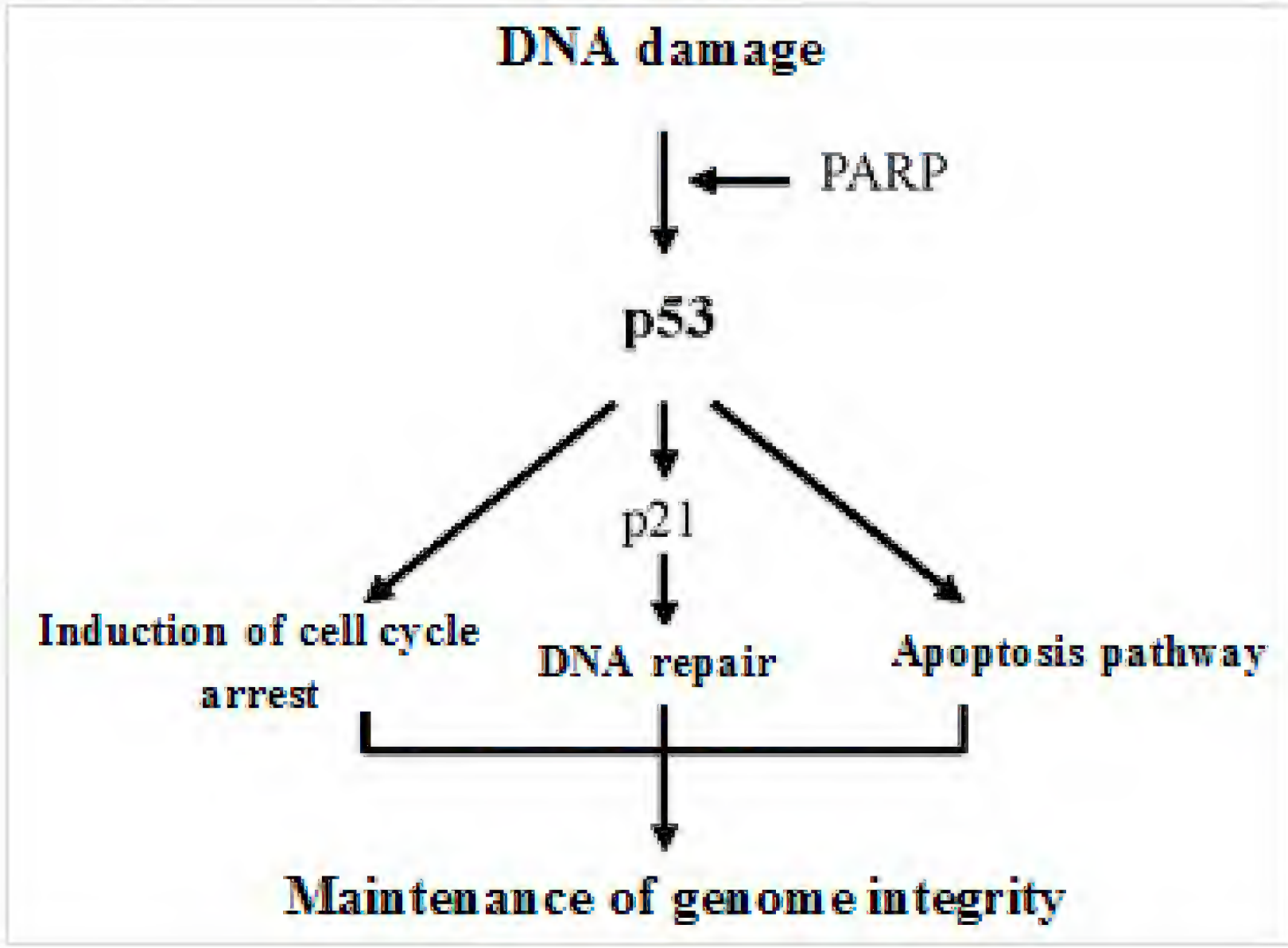

Figure 17. Roles of PARP1 in activating the p53-related pathways and the maintenance of the genome integrity.

Poly(ADP-ribose) is known to bind and modify various DNA damage checkpoint proteins – p53 and p21(CIP1/WAF1), proteins associated with repair of damaged DNA, such as XPA, MSH6, DNA ligase III, XRCC1, DNA polymerase ε, DNA-PK, Ku70, and other signalling and effector molecules, among which are NF-κB, inducible nitric-oxide synthase, caspase-activated DNase, and telomerase [899, 900]. Poly-(ADP)-ribosylation may prevent and reverse binding of p53 to its consensus sequences in the transactivated genes and to single-strand end in DNA [901].

Self-poly-ADP-ribosylation of PARP1 results in decreasing its affinity to DNA (inactivation). Accumulation of inactivated PARP in the cell causes depletion of the cellular NAD^+, and, subsequently, ATP. Lack of ATP in the cells would, however, eventually lead to necrotic death because of energy depletion. Therefore, in pre-apoptotic cells PARP1 is proteolytically degraded by caspase-3 in order to avoid cell necrosis [902,903]. Accumulation of poly-ADP-ribose polymers in a cell is usually a signal for activation of the programmed cell death pathway [904].

Poly-(ADP-ribose)-polymerase 1 activity was found to play a crucial role in mammalian meiosis and gamete selection through apoptosis [892,905,906]. In mice, Parp1 activity has been found to be essential for normal spermatogenesis [907]. During spermatogenesis, the reorganisation of a diploid, histone-associated genome into a haploid, predominantly protamine-associated genome is accompanied by occurrence of physiological DNA strand breaks, which exist only temporarily [908]. Parp1, Parp2, and probably other proteins of the PARP family play important roles in chromatin remodelling during spermatogenesis, and

deficiencies in poly-(ADP)-ribosylation are currently suspected as causes for infertility in mammals because of histone retention during spermatogenesis, resulting in immature sperm chromatin [909]. 3-aminobenzamide, a known PARP1 inhibitor, was found to have a protective effect against experimental immune ovarian failure in mice, probably by decreasing the levels of necrotic cell death in follicular cells [910].

Mouse models carrying inactivated copies of the *Parp1* gene exhibit accelerated telomere shortening compared to wild type mice [911].

PARP2 seems to play a role in the stability of the X-chromosomal stability. Among Parp2 knockout mice, female pups were born at a lower frequency than expected, and cytogenetic analysis revealed chromosomal instability and increased rates of intrauterine death in female embryos [912].

More than 20 years ago, PARP1 activity in mononuclear cells from peripheral blood was found to correlate positively with the maximal life span in mammals [913]. PARP1 was identified to play a role in normal ageing, probably through regulation of DNA break repair, apoptosis and necrosis [summarised in 914]. PARP1 activity and PARP1 content generally declines with age, but it was reported to be higher in cells from centenarians than in controls [915]. More recently, it has been reported that OGG1 and NEIL1, two of the mammalian base excision repair glycosylases, bind to PARP1 and stimulate its activity [916,917].

PARP1 has been identified as a therapeutic target in cancer, with its inhibition intended to decrease the capacity for DNA repair in cancer cells damaged by radio- and chemotherapy [reviewed in 918]. Several PARP1 inhibitors (PJ34, NU1025, and others) are currently under study as sensitising agents in cancer, other are completing clinical trials (AZD2281 (olaparib), rucaparib, veliparib and others) [919-921].

The carriership of some polymorphic variants of PARP have been found to play a role in the constitution of the risk for development of chronic graft-versus-host dis¬ease after transplantation of allogeneic haematopoietic stem cells [215,216]. A polymorphism in a processed pseudogene of *PARP1*, located on chromosome 13 (13q33) was reported to be associated with increased risk for several common cancers [922].

5.9. DNA-dependent protein kinase

DNA-dependent protein kinase (DNA-PK, also DNA-activated protein kinase) is a nuclear serine/threonine kinase playing a role in the signalling pathways related to DNA repair, and specifically in non-homologous end joining [923,924]. The latter includes not only NHEJ for the purposes of DNA repair, but also the class switch recombination in immune cells [925,926].

The functional DNA-PK protein is made of two subunits – the catalytic subunit of DNA-PK (PRKDC) and a composite regulatory subunit – the Ku protein. Ku protein is made of two polypeptide chains (Ku70 (p70) and Ku80 (p80), respectively, with molecular mass of approximately 70 and 80 kDa). It was first described in as an autoantigen in systemic lupus erythematosus [927,928]. Ku antigen has double-strand DNA binding activity without sequence specificity. Ku70 and Ku80 exhibit ATP-dependent helicase activity on single-strand DNA [166,167].The catalytic subunit of DNA-PK is inactive without the Ku antigen and needs to be bound to it for activation of its catalytic activity [925,929].

The gene coding for the catalytic subunit (*PRKDS*) is located at 8q11.21. The genes coding for the two polypeptide chains of the Ku antigen are, respectively, *XRCC6* (22q13.2, coding for p70) and *XRCC5* (2q35, coding for p80). The Ku70/Ku80 dimer acts as regulatory subunit of DNA-PK, increasing the affinity of the catalytic subunit PRKDC to DNA by a factor of 100 [167]. Mice in which both copies of the gene coding for the catalytic subunit of DNA-PK were inactivated exhibited severe combined immune deficiency and sensitivity to ionising radiation [930]. Mice with defects in the genes coding for the Ku70 polypeptide chains of the Ku antigen exhibit a cancer-prone genotype characterised by higher-than-normal levels of sister chromatid exchange and high frequency of spontaneous chromosomal breakage and translocations [931,932].

DNA-PK is activated in the presence of free reactive ends in DNA (double-strand breaks) [933]. Upon DNA damage, it phosphorylates Ser139 of the histone variant H2AX, activating the associated DNA damage response pathway. DNA-PK also phosphorylates histone H1; PARP1; XRCC1; XRCC4 (the complement of ligase IV); DNA crosslink repair protein 1C (Artemis); the Werner syndrome helicase WRN; products of cellular proto-oncogenes (c-MYC, c-JUN, c-ABL1) and others [335,934,935]. In response to DNA damage, DNA-PK is capable of phosphorylating directly p53 on selected serine residues (Ser15, Ser37), which interferes with MDM2-mediated tagging of p53 for degradation, thus allowing p53 accumulation in the cell [936]. Similarly to PARP proteins, DNA-PK is capable of automodification (in this case, autophosphorylation) on the catalytic subunit as well as on the Ku protein components [937,938].

The loss of expression of Ku70 in HPV-infected cervical epithelium was recently found to be associated with increased risk for progression to cervical intraepithelial neoplasia (CIN) [492]. Inherited defects of DNA-PK are rare in man, presumably because of high risk of early loss of affected embryos. In 2009, however, a missense mutation in the human gene coding for the catalytic subunit of DNA-PK was identified in a patient with severe combined immune deficiency [939]. Inherited defects in DNA crosslink repair protein 1C (Artemis), activated by phosphorylation by DNA-dependent protein kinase, also produce a phenotype of combined immunodeficiency [335,437]. Functional impairment of DNA-PK (downregulation of the expression of the catalytic subunit, coupled with upregulation of the expression of the Ku protein) was found in polycythemia vera [940].

The possibilities for targeting specifically DNA-dependent protein kinase in tumour cells in order to increase their sensitivity to chemo- and radiotherapy are currently being explored. Inhibition of DNA-PK by the inhibiting compound BEZ235 was found to induce accelerated senescence in cancer cells treated with ionising radiation [941]. Induction of cell cycle arrest in G2/M phase was demonstrated in cancer cells treated with etoposide or ionising radiation and the DNA-PK inhibitor NU7441 [942]. It has been found that suppression of DNA-PK may enhance growth factor-dependent cell proliferation in endothelial cells, thus suppressing angiogenesis [943].

The levels of wild type and cancer-specific DNA-PK may be used as markers in prognostication of outcomes in some types of leukemia, with high DNA-PK levels associated with reduced treatment-free interval [944].

5.10. Retinoblastoma protein

The *RB1* gene (13q14.2), coding for the retinoblastoma protein (pRB1) was the first human tumour-suppressor gene to be cloned [945]. The *RB1* gene is part of a gene family, comprising proteins with various functions in regulation of transcription, maintenance of chromatin structure and negative control over the progression in the cell cycle (pRB, p130 and p107) [946,947]. The coding sequence of *RB1* is significantly conserved among primates and humans [948].

pRb protein (in humans, pRB1) is localised predominantly in the nucleus. It acts as an inhibitor of the G1-S phase progression by binding and inactivating the transcription factor E2F (E2F1) (Fig. 14) thus repressing the transcription of crucially important S-phase genes [949,950]. More specifically, E2F1 is a member of a family of DNA-binding proteins (as heterodimers with other proteins, termed E2F dimerisation partners or DP) that regulate the transcription of several proteins needed in DNA replication [951-953]. In intact cells, the binding of E2F1 to its response elements usually stimulates cellular proliferation, but in the presence of DNA damage it may promote p53-dependent apoptosis [954]. In stressed cells, the activated checkpoint kinase CHK2 phosphorylates and activates E2F1 in response to DNA damage [955]. Activated E2F1 typically works by transcriptional activation of proteins inactivating and/or sequestering the MDM2 ligase, allowing p53 accumulation in the cell [956]. Wild type p53 suppresses the transcription of pRB1 [957].

pRB1 is extensively modified by cyclin/CDK complexes in the G1-S phase transition in the cell cycle. In G0/G1 cells virtually all the pRB1 is unphosphorylated, during S and G2 phases; it is predominantly phosphorylated, to be dephosphorylated in late M phase [958]. pRB1 is phosphorylated by cyclin C/CDK3 in cells exiting from the G0 phase [959]. To release E2F in late G1, so as to ensure transition to S phase, pRB1 is inactivated by sequential phosphorylation at selected serine residues by activated cyclin-dependent kinases CDK6, CDK4 and CDK2 [831,832].

Deletion of exons 13–17 of the *pRB1* gene is frequently observed in tumours – e.g. in retinoblastoma, breast cancer, osteosarcoma and small-cell lung cancer [960,961]. Deregulation of pRB1-controlled pathways is a common feature in various cancers, heritable and sporadic, including cancers with viral pathogenesis (e.g. HPV-related precancerous lesions and overt cancer) [949,962,963].

6. DNA repair and programmed cell death

You came in (death) without any compulsion
or pushing, of your own free will.
Lucian of Samosata, Dialogues of the Dead, XXI.
(c. II century A.D).

6.1. Basic concepts

Programmed cell death (apoptosis) allows for planned, controlled and irreversible termination of all vital functions of the cell and its physical removal from the cell pool. Every cell in multicellular organisms is equipped with a complex apparatus intended specifically for the implementation of programmed cell death. It may be triggered by many different

stimuli, of exogenous as well as endogenous origin, and usually the one complements the other.

The decision whether the damaged cell should live or die is taken individually, depending on the particular circumstances and is based on assessment of the scale of the damage [649,964]. The process of decision-making is tightly regulated and controlled at multiple levels, involves various checking mechanisms and complicated signalling pathways [summarised in 965 and 966].Usually, damaged cells are routed to the apoptotic pathway in the G1-S checkpoint of the cell cycle. If the damage is assessed as potentially reparable, the S phase is delayed until the DNA is recovered to the state it was before the damage occurred (or as close to it as possible). In cases when damage is too extensive and/or too severe, or the cell has been assessed as a danger to other cells (e.g. has been infected with a virus or other intracellular parasite; has begun cancerous transformation; is under imminent threat of dying by necrosis, damaging other cells in the tissue, etc.), it is directed to the apoptotic pathway. In the rare cases when the very mechanism of assessment of the damage is impaired (e.g. in inherited ATM deficiency), cells are unable to detect small-scale DNA damage and would not halt cell cycle progression and attempt repairs, but would continue dividing until the cumulative level of damage reaches a certain critical threshold, beyond which the programmed cell death routine is launched automatically. The working principle of many modern anticancer medications is based on this mechanism of induction of apoptosis in cancer cells [967].

Once the final decision about the fate of the cell is made, however, the process becomes irreversible. Special care is taken that the genetic information in the dying cell is completely destroyed before it actually dies. The apoptosis mechanism ensures that all the phases of the death of the cell are carried out in an orderly and systematic fashion (so that there is not even the slightest chance that a cell destined to die might actually survive), and that it is implemented in a manner that is safe for the other cells (unlike other types of cells death, in which the ruptured or leaky cell membranes let the contents of the dying cell spill out, triggering local and/or systemic reactions).

Programmed cell death is frequently used mechanism in normal tissues – as part of the normal cellular turnover for removal of aged or damaged cells; for regression of rudimentary organs or tissues and organs existing only temporarily (e.g. during the embryonic or larval development); or simply for the purposes of control of the number of cells in a tissue or organ, so as not to cause them to grow or shrink disproportionately to the size and mass of the organism. The regression of the tails of tadpoles during their metamorphosis into adult amphibians and the disappearance of the webbing of the fingers and toes of human embryos around 8th gestation week are among the most commonly cited examples of physiological apoptosis of large cell populations. Another, less commonly known example is regression of drug-induced liver hypertrophy in mammals after withdrawal of the offending drug. Treatment with certain drugs such as phenobarbital stimulates the proliferation of hepatocytes, resulting in enlargement of the liver and spleen. After the treatment is terminated or the dose of phenobarbital is decreased, massive apoptosis takes place, removing the surplus cells, and the liver quickly shrinks to its normal size and mass.

Programmed cell death usually occurs in single cells and specific cell populations, but may also be implemented on a larger scale (the whole organism), if the individual programme of the organism dictates that it must be done. In some species of lower animals developing by metamorphosis (usually, insects – moths, mayflies, cicadas, etc., referred to as ephemeral insects), the sexually mature form (the imago) would only live from several minutes up to several weeks, depending on the species. The imago exists solely for purposes of procreation and may even be devoid of functional mouth apparatus. After the allotted time expires, the imago dies quickly, within hours or minutes, regardless of whether it has managed to procreate or not. This, however, is not 'death of old age' as we know it, as the cells and the tissues of the dying insect are usually in working order. Neither is it simple depletion of energy sources, as autophagy may sustain the organism beyond this time. Death in ephemeral insects usually occurs because of rapid time-coordinated apoptosis of cells in vital tissues and organs, dictated by the genetic programme of the species. In plants with limited lifespan (annuals – e.g. Arabidopsis thaliana, rice, maize, etc.) controlled fragmenting of the chloroplast DNA occurs after the leaves have reached maturity and several hours to several days before the chlorophyll is degraded (manifested by yellowing of leaves) [968,969]. DNA fragmentation is believed to be one of the signals triggering the ageing process in the cells of the plant [970,971].

Apoptosis is a default option for some cells at certain stages of differentiation, regardless of the absence of damage to the cell. In lymphocyte differentiation, for example, all cells that do not comply with the requirements of the differentiation stage and have not received special 'survival signals' will automatically be routed to the apoptosis pathway, despite the fact that they are not stressed or damaged in any way.

In some cases, imminent apoptosis may be delayed in time (but not prevented altogether). For example, the milk-producing cells in the mammary glands of lactating female mammals may live and function for different periods of time (from several days or weeks to months and even years) before they are eventually directed to apoptosis. The mammary gland contains extensive network of branched ducts, paved with epitheloid stem cells on the inside and insulated with connective and fat tissue on the outside. In non-pregnant, non-lactating female mammals the mammary glands are quiescent and the resident stem cell population is sparse and replicatively inactive. During pregnancy, the hormonal stimulation of the gland stimulates the division of the stem cells, eventually producing two types of differentiated cells –secretory cells (producing the milk) and myoepithelial cells (capable of active contraction, facilitating the expression of the milk). As long as the mammary gland is regularly stimulated to produce milk (by breastfeeding or expression), the differentiated cells will live and function, and will be replenished with new cells when necessary. This may continue for a very long time (in some cases, up to several years) after the gland is no longer stimulated, however (e.g. the baby is weaned off the breast), the milk secretion dries up relatively quickly, in a matter of days. This occurs via mass apoptosis of the differentiated cells in the gland and is apparently dependent on the presence of unexpressed milk rather than on the hormonal status, as it occurs in a matter of hours and days, while hormonal status is not normally subject to rapid changes. The mammary glands of the post-lactating

female regress back to their quiescent state, with only the epitheloid stem cells surviving the purge, to be reactivated in the next pregnancy.

Programmed cell death is very unlike the death resulting from acute damage to the cell (necrosis) or targeted destruction of the cell (cell lysis). The enzyme activities in the apoptotic cell are targeted inwards, at the cell itself, digesting its own contents. Necrotic cells usually release their cytoplasmic contents out onto the adjacent cells, together with the unprotected hydrolytic enzymes. Cells dying by apoptosis do not activate the immune system of the organism, while necrotic cell death usually results in inflammation. Apoptosis may occur in cell populations, but can be controlled to the level of a single cell, while necrosis usually affects larger groups of cells, even tissues and organs. Finally, necrotic processes usually stimulate tissue regeneration while apoptosis will not normally initiate any regenerative processes, as the cells that were lost need not be replaced immediately.

6.2. Brief history of the discovery of the phenomenon of programmed cell death

Apoptosis was discovered relatively early, in mid-XIX century, by the German zoologist Karl Vogt, who studied the reduction of the tails of tadpoles in amphibian development. There was not much interest in such a subject at the time (as it was not considered 'useful'), so the studies of Vogt were practically forgotten until more than a century later.

In the late 60-ties and the 70-ties of the XX century, the studies in the field of programmed cell death were renewed with the works of the group of the Australian pathologist John Kerr at the University of Queensland, on the regeneration of rat liver after acute injury [972,973]. Initially Kerr and colleagues named the phenomenon they have been observing 'shrinkage necrosis' or 'programmed cell necrosis'. Later, after consulting Prof. James Cormack from the Department of Greek at the University of Aberdeen, UK, the term 'apoptosis' was approved (literally meaning 'shedding petals or leaves (off flowers, trees, etc.) Approximately at the same time as the studies of Kerr, the Scottish pathologist Alastair Currie and his PhD student Andrew Wyllie reported their observations of membrane-limited cell fragments containing condensed chromatin in tissues subjected to damage [974].

The signalling pathways of apoptosis and the participating molecules were first identified and described about 30 years ago, in studies of the life cycle of the microscopic nematode Caenorhabditis elegans [975-977]. Several years after the initial discoveries of the proteins responsible for apoptosis of a fixed number of cells during the individual development of C. elegans, the first mammalian homologues of apoptotic genes and proteins were identified [978-980].

At present, the number of published papers about apoptosis is close to 300,000 – that is 1.5 times more than papers of DNA replication and only two times lower than the number of papers dedicated to transcription.

6.3. Distinguishing features of apoptotic cells and stages in apoptosis

Cells dying by apoptosis exhibit several distinctive traits that make them very different from the surrounding living cells. Apoptosis affects the cell morphology as well as its biochemical and metabolic properties. Early in apoptosis, the dying cell loses its capacity for adhesion to the extracellular matrix and the adjacent cells and it becomes more and more rounded as the interaction with the cell milieu progressively diminishes. The pH of the mitochondrial matrix becomes more alkaline, while the cytosol is acidified. This is believed to create a

favourable environment for the action of caspases [981]. The proteins controlling the intracellular redox potential (e.g. the thioredoxin and glutathione systems) are up-regulated [982]. The cell loses some of its water content and its volume decreases. The ordered structure of the cytoskeleton disaggregates, resulting in appearance of bulging deformations (blebs) in the plasma membrane. The cisterns of the endoplasmatic reticulum become dehydrated and form fluid-filled vacuole. The cellular endonucleases are activated and released within the cell, resulting in fragmentation of the nuclear chromatin and the typical 'ladder image' seen in electrophoresis of DNA extracted from apoptotic cells (Fig. 18). Later, the nucleus also becomes fragmented (caryorrhexis).

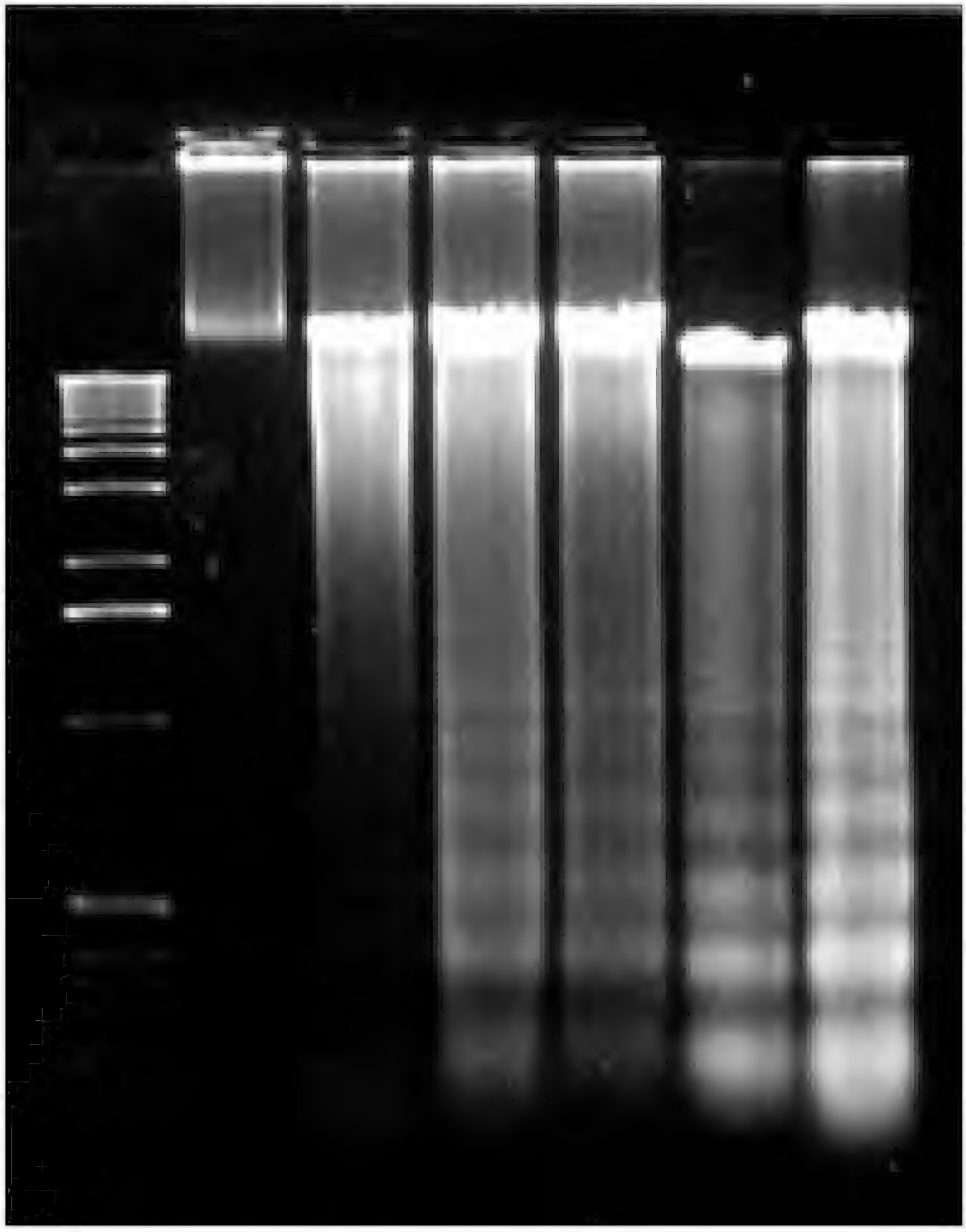

Figure 18. "Ladder image" of DNA in apoptotic cells (lanes on the right) in an agarose gel stained with ethidium bromide. For comparison, intact DNA is loaded on the second lane from left to right [983].

In the late stages of apoptosis, the cell disintegrates into several small cytoplasmic masses with rounded contours (apoptotic bodies), packed in plasma membrane and containing the nuclear fragments and the remainders of the autophaged cellular organelles. Eventually, the apoptotic bodies are taken up by phagocytosis by the resident macrophages in the tissue and are degraded completely (Fig. 19).

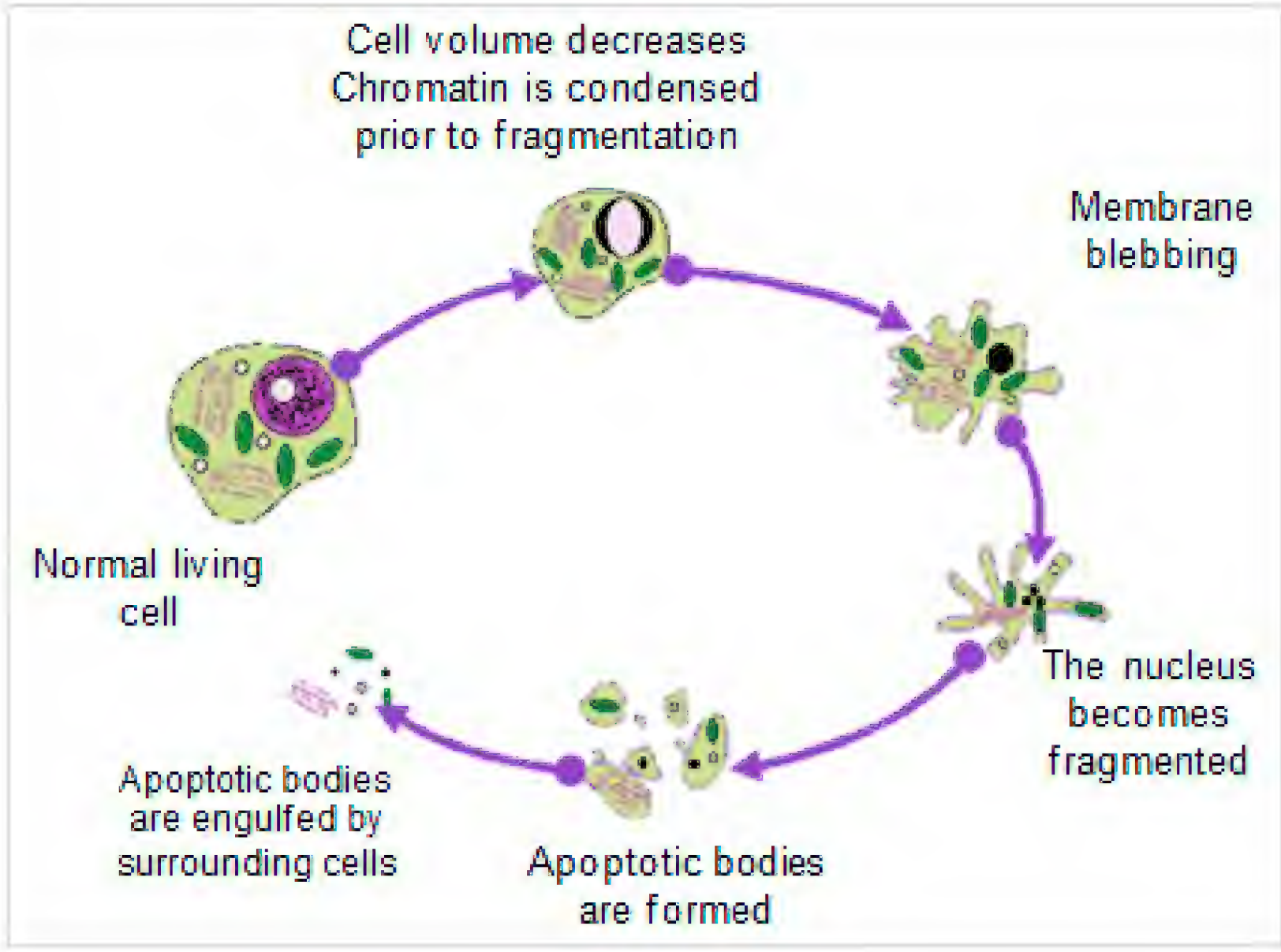

Figure 19. Major phases in apoptosis.

Programmed cell death proceeds rapidly and may be complete in a matter of hours. Crude estimates show that the pace of apoptosis of cells in multicellular organisms is about 20 times faster than the pace of cell division. In mammals, every day several dozens of billions of cells die by apoptosis. In some tissues (e.g. skin, mucosa, bone marrow) the natural cellular turnover is high, in others (e.g. CNS neurons, muscle tissue) it is naturally low, but according to the calculations of Melino, if cellular division was not compensated by apoptosis, at the age of 80, the average man would carry around about 2000kg of bone marrow and over 16 km of intestines [984].

The genetic information of the dying cell is destroyed beyond repair and the tools to make it recoverable (signalling and effector molecules of DNA repair) are rendered unusable. This an additional mechanism for making the decision for apoptosis irrevocable and the process impossible to reverse – it may be delayed for some time, but once it has started, it cannot be halted, reversed, rerouted to an alternative pathway, or made to deviate from the pre-planned schedule.

6.4. Major signalling and effector molecules in apoptosis

6.4.1. General outline of apoptotic mechanisms

Caenorhabditis elegans

C. elegans is virtually the ideal model organism for studies of programmed cell death, as the adult organism is made of a fixed number of cells. C. elegans has two sexes –

hermaphrodites and males. The number of cells is different between the two sexes and may vary between different strains, but remains fixed for the particular strain and for the particular sex. During the development of C. elegans hermaphrodite, a total of 1090 cells are generated, of which exactly 131 cell die by apoptosis. Apoptotic cells exhibit different morphology from the other cells and refract light in a different manner, so in a small and virtually transparent organism such as C. elegans they may be observed and monitored in vivo. 113 of these cells die during embryonic development and 18 – during post-embryonic development [985,986]. The body of the adult hermaphrodite nematode is made of exactly 959 somatic cells (Fig. 20).

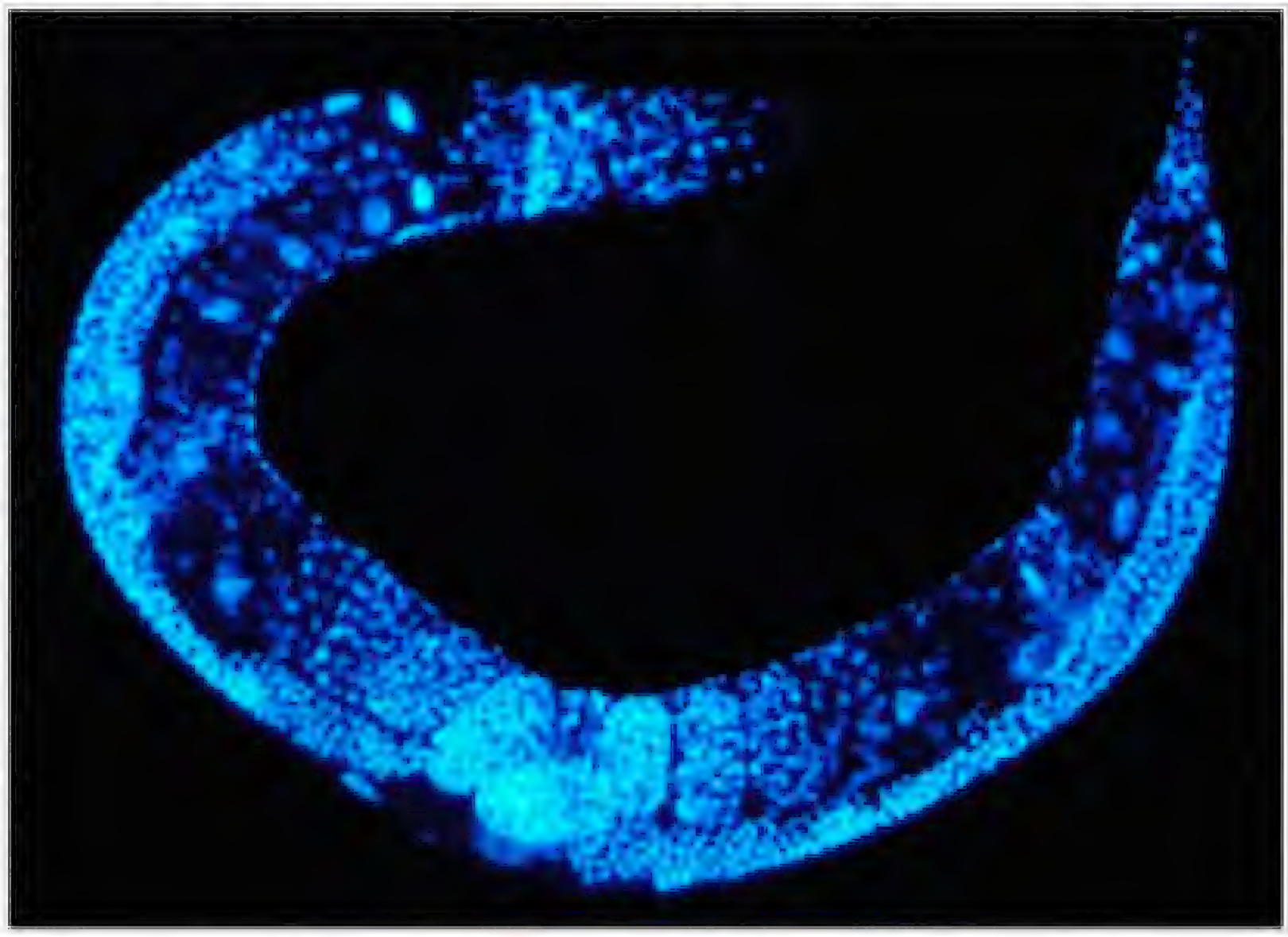

Figure 20. An adult C. elegans hermaphrodite. The nuclei of all cells are stained with 4',6'-diamidino-2-phenylindole hydrochloride (DAPI) [987].

It is notable that the implementation of the apoptotic death of these 131 cells is not vitally important for the nematode (at least, adverse events related to absence of physiological apoptosis in C. elegans have never been observed in laboratory settings). If the apoptosis of the selected cells is not promptly carried out, the worm will live and develop quite normally, only it would have more cells in the body than usual. Usually, the 'surplus' cells differentiate along the neural lineage [988].

Apoptosis in C. elegans is carried out in three phases, termed specification, killing, and execution stages, respectively [989]. The specification stage is the stage in which the cells that are destined to apoptosis are prepared and instructed for it. The killing stage involves initiation of the apoptosis cascades. In the execution stage the chromatin is fragmented, the nucleus and the cell disintegrate and the resulting apoptotic bodies are finally engulfed by the adjacent cells.

The specification stage is regulated by several transcription factors, products of the nematode genes *eor-1* and *-2*, *ces -1* and *-2*, *hlh-2* and *-3*, and *tra-1*. The proteins hlh-2/hlh-

3, ces-1 and tra-1 are believed to regulate the transcription of egl-1, a factor specific to the stage of killing [990,991].

Several of the key proteins acting in the regulation of the killing and execution stages in apoptosis in C. elegans are coded by the ced genes family (cell death abnormal) – namely, *ced-1*, *ced-2*, *ced-3*, *ced-4* and *ced-9*. Apart from them, there are other proteins with important roles in C. elegans apoptosis as well, such as the already mentioned egl-1, the products of the *crn* nuclease family, the *ces* gene family, and others.

Three genes are chiefly responsible for the killing stage in the physiological apoptosis in C. elegans – *egl-1*, *ced-3* and *ced-4*. *egl*-1 (*egg-laying defective 1*) codes for a low molecular weight protein, carrying a specific BH3 peptide motif (Bcl-2 homology region 3, a motif typical of the pro-apoptotic proteins of the *Bcl-2* gene family) [992].The ced-9 protein has anti-apoptotic properties [977,993]. egl-1 activates the mechanism of programmed cell death by binding to ced-9 and inactivating it [994]. ced-3 and ced-4 are positive regulators of apoptosis in C. elegans. Deficiency or impaired functions of either protein result in abolition of developmental apoptosis in C. elegans [988]. It is notable, however, that mRNA of ced-4 may sometimes splice differently to produce an alternative transcript that is capable of inhibition of programmed cell death [995].

Destruction of the DNA of the dying cell by activation of cellular nucleases is a crucial step in apoptosis, ensuring that there is no chance that the cell might escape from the suicide pathway. In the execution phase, shrinkage of the cytoplasm and fragmentation of the chromatin occurs. In C. elegans, the major proteins playing a role in the final phase of apoptosis are nucleases (nuc-1; cell death related nucleases (crn) 1–6; mitochondrial nucleases such as cps-6) but also other enzymes with roles in the autophagy of cell contents [996-998].

The dying cell exports specific proteins to its outer membrane, serving as signals for the other cells to engulf its disintegrated remains (commonly called 'eat me' signals). Among the latter are the proteins ced-1, -2, -5, -6, -7, -10, -12 and psr-1 (phosphatidylserine receptor homologue) [999]. These proteins act in two partially overlapping signalling pathways, with ced-1, -6 and -7 in one pathway and ced-2, -5, -10, -12 and psr-1 in the other. *ced-1* and *-2* were the first genes in which mutations, related to disorders in apoptosis in C. elegans were identified. Inactivating mutations in these genes produce accumulation of dead 'uneaten' cells [1000].

Mammalian homologues of the apoptotic genes of C. elegans

Over 10 mammalian homologues of ced proteins of C. elegans have been identified so far [978-980,1001]. For example, human *APAF-1* gene (coding for apoptotic protease activating factor-1, activator of caspase-9) is a homologue of the C. elegans *ced-4* [1002]. The human proto-oncogene BCL-2 is a homologue of C. elegans *ced-9* gene [993]. The signalling caspase-1 and the effector caspases -3, -6 and -7 (beta-transcript form) are mammalian homologues of ced-3 of Caenorhabditis elegans [1003-1006]. PTB domain-containing engulfment adaptor protein 1 (GULP1) is the human homologue of ced-6, a C. elegans protein functioning in the assimilation of the digested remains of apoptotic cells [1007]. Besides the homologues of ced proteins, higher animals also employ many other proteins with pro-apoptotic and anti-apoptotic properties (see below).

6.4.2. Caspases and other proteins with functions in apoptosis

Apoptotic pathway may be activated by stimuli of exogenous origin (receptor-mediated or extrinsic mechanism) or of endogenous origin (mitochondrial-dependent or intrinsic mechanism). In either mechanism, the central functions are implemented by specific proteins termed **caspases** (**c**ysteine-dependent **asp**artate-directed prote**ases**). 'Caspase' is a trivial name, of course, but it has become very popular. In fact, not many people know that, for example, caspase-1 is the same as interleukin-1-beta-convertase or ICE, caspase-3 – PARP cleavage protease (also known by other trivial names – apopain, or Yama), etc.

Caspases are cysteine proteases catalysing the hydrolysis of other proteins at designated aspartate residues [1008]. The result is usually activation of the target protein. Caspase activity is typically regulated at post-translational level, allowing them to be activated rapidly in response to specific triggers.

Depending on their targets, different caspases may be divided in two major types – signalling and effector caspases. Signalling caspases activate other downstream-acting caspases, relaying and amplifying the pro-apoptotic signal. Effector (executor) caspases catalyse the proteolytic cleavage of various key substrates, causing release of nucleolytic and proteolytic activities in order to disintegrate the nuclear lamina, destroy higher-order chromatin structures, degrade the DNA of the cell and digest the cell's contents. Caspases also cleave and inactivate key proteins of DNA damage-related signalling and repair. Activation of initiatory caspases launches a signalling cascade that amplifies the initial pro-apoptotic signal, so that apoptosis is rapidly carried to completion [902,1005,1009].

In the living cells there is always a pool of caspases circulating in an inactive form (pro-caspases, zymogens). Pro-caspases contain a pro-domain, a larger subunit carrying the catalytic activity, and a smaller subunit. The pro-domain usually plays a role in caspase activation (see below). Activation of caspases is carried out by hydrolysis at a specific aspartate residue, usually by other, upstream-functioning caspases [1010]. The activation of pro-caspases separates the two subunits in the mature caspase, thereby greatly enhancing its proteolytic activity. The active caspase unit is usually a tetramer made of two large and two small subunits [1011]. Activated caspases are capable of activating, in their turn, other pro-caspase molecules, generating a proteolytic cascade amplifying the initial pro-apoptotic signal. Among the major proteolytic targets of the apoptotic cascade are nuclear lamins, histones and other chromatin proteins, and enzymes acting in damage signalling and DNA repair (ATM, DNA-dependent protein kinase, poly-(ADP-ribose)-polymerase (PARP1) protein, and others) [reviewed in 1012]. This prevents initiation of DNA repair (and potential survival) of a cell that was destined to die.

Different caspases recognise different peptide motifs in their target proteins. Some of these motifs are recognised by a single, unique caspase, others may be recognised by several caspases [1013-1015].

The initiation of the pro-apoptotic cascade is induced with the aid of specific adapter proteins that work by recruiting and assembling many initiator pro-caspase molecules together so as to form a pro-apoptotic complex (aggregate). There is a specific term for this – 'apoptosome', referring to the complex of adapter proteins mediating the activation of initiator caspases. It is believed that single pro-caspase molecules have but weak proteolytic

activity of their own. The formation of the pro-caspase complex is accompanied by conformational changes in their molecules. Eventually, activation occurs, with every molecule cross-activating the other molecules in the aggregate (induced proximity model of activation) [1016-1017]. Once the pool of initiator caspases has been activated, they are capable of activating downstream acting effector (executor) caspases. The mechanism of activation of an executor caspase is somewhat different from the activation of the initiator caspase. Specifically, it involves conformational changes in the active site of the zymogen, facilitating substrate binding and hydrolysis [1018-1019].

Some caspases are highly conserved between different species (e.g. the homologues of ced proteins), others are specific for certain groups of organisms only. For example, until recently, caspase-4 and caspase-5 have been considered to be found only in humans [1020]. Later, it was found that murine caspase-11 was, in fact, a homologue of human caspase-4 [1021]. The amino acid sequences of caspase-4 and -5 is similar to one another and to the sequence of caspase-1, the three loci having probably arisen by tandem duplication of an ancestral locus in the 11q22 band [1022]. Rodent caspase-12 is normally active, but in about half of the humans at least one of the gene copies is inactive because of a premature stop codon [1023]. The prevalence of the inactive gene copy of caspase-12 in human populations is different in different ethnic groups, with 20–30% of individuals of African origin and virtually 0% in Europe and Asia carrying the active gene form [1024]. An association between the carriership of the inactive gene copy of *CASP12* and resistance to sepsis was elicited several years ago. It is believed that the inactivation initially occurred as a result of random mutagenesis, but had become fixed because of positive selection [1025]. Caspase-13 has so far only been identified in cattle [1026].

A list of major mammalian caspases and a summary of the functions in apoptosis and in other biological processes are presented in Table 5.

Table 5. Biological roles for major caspases in mammals 1027-1030].

Caspase Nr	Role in apoptosis	Other roles
1	Initiation of apoptotic cascade	IL-1 production Cytokine maturation Myoblast differentiation Cell migration NF-kappaB activation
2	Initiator or executor caspase	Differentiation of precursor cells along the myeloid lineage -erythroblasts, macrophages and osteoblasts DNA repair
3	Executor caspase	Differentiation of precursor cells along the myeloid lineage – erythroblasts, macrophages and megacaryocytes Differentiation of osteoblasts, keratinocytes, myoblasts, epithelial cells, cells of the ocular lens and neural stem cells Trophoblast differentiation (in embryonic development) Inhibition of B-lymphocyte proliferation IL-16 production

4	Still not very well characterised Activation of caspase-1	Inflammation Modulation of innate immunity via caspase-1
5	Initiation of apoptotic cascade	Maturation of pro-inflammatory cytokines
6	Previously believed to be an executor caspase Cleaves and activates pro-caspases -2, -3 and -8	Ocular lens fiber differentiation Stimulation of B-lymphocyte proliferation
7	Executor caspase	Differentiation of the erythroid lineage
8	Initiator caspase with DE-domain	Proliferation of T-, B- and NK- cells Trophoblast differentiation (in embryonic development) Differentiation of precursor cells along the myeloid lineage –erythroblasts, monoblasts
9	Initiator caspase, activating the apoptosis by the endogenous mechanism	Same as caspase-3, also differentiation of epithelial cells
10	Initiator caspase with DE-domain	NF-κB activation
11	Initiator caspase with DE-domain	Maturation of pro-inflammatory cytokines
12	Initiator caspase	Anti-inflammatory properties Susceptibility to sepsis
14	At the moment considered to be a non-apoptotic caspase	Differentiation of keratinocytes in cornifying epithelium

Initiator caspases may be further sub-classified with regard to their targets into activators of cytokine-related signalling (caspases -1, -4, -5, -11 and -13) and initiating caspases of the apoptotic executive cascade (caspases -2, -8, -9, -10 and -12). Another type of classification of initiator caspases is with regard to their major protein domains. According to this, initiator caspases are divided into caspases containing a DE domain (death effector domain, DED) and caspases containing the CARD domain (caspase recruitment domain). The former type of caspases contains a specific domain with affinity to membrane receptors of the type of tumour necrosis factor receptor superfamily, such as TNF-alpha receptor or FAS (apoptosis antigen-1 or CD95), or others (e.g. TRAIL receptors). DED initiator caspases are activated by binding of specific ligands (death ligands) to the respective membrane receptors (death receptors), forming a death-inducing signalling complex (DISC). As a result, receptor clustering and death domain aggregation is induced, then the ligand-receptor complex is internalised. Inside the cell, an adapter protein binds to the receptor molecule, linking recruiting the initiator caspase-8. Once bound, caspase-8 molecules activate each other and downstream caspases and other target molecules by partial proteolysis, launching the apoptotic cascade.

The other major type of initiator caspases are those containing the CARD domain. Such are, for example, initiator caspases -2 and -9. CARD-domain caspases are recruited to the initiation receptor-caspase complex via RAIDD adapter proteins [1031, 1032]. RAIDD are adapter proteins possessing a death domain, allowing binding to various signalling proteins, such as PIDD (p53-induced protein with death domain, a pro-apoptotic protein capable of activating caspase-2) and cytokine signalling proteins, such as FasL/tumour necrosis factor receptor-interacting protein (RIP), and a CARD-domain for binding to caspase-2

[1033,1034]. Thus, caspase-2 molecules are brought close enough to proteolytically activate each other.

Caspase-9 is also activated via its CARD-domain in an ATP-dependent process, in the presence of the factor APAF-1 (see below) and cytochrome c [1035,1036].

Caspases -3 and -7 are considered to be the major executor caspases in mammals. Their action is chiefly responsible for the process of condensation of the chromatin of the dying cell and its subsequent fragmentation 1037,1038]. Caspase-6 was originally classified as an executor caspase, but later it was found that its substrates were pro-caspases-2, -3 and -8, therefore, it is, strictly speaking, an initiator caspase [1039,1040]. The first activated executor caspase is usually caspase-3 (by initiation caspases -8, -9 and -10), which, in turn, activates caspase-7.

Effector caspases are usually activated by initiator caspases, but some proteins may activate effector caspases by an independent mechanism. Such is, for example, granzyme B (cytotoxic T-lymphocyte-associated serine esterase 1; CTLA1). Granzyme B is a serine protease characteristic for natural killer (NK) cells and cytotoxic lymphocytes. It plays a crucial role in the induction of apoptosis in target cells by direct activation of executor caspases -3 and -7 [1041-1043]. Some of the members of the Bcl-2 protein family may also activate apoptosis independently [1044].

Cytokine deprivation may induce apoptosis in cultured mammalian cells by a pathway independent of death receptors-mediated signalling. This involves upregulation of the transcription of the CDK inhibitor p27 (KiP) and proteins with pro-apoptotic properties, such as Bim (of the BCL2 12family) [1045]. Also, cytokine withdrawal may induce cytochrome c release from mitochondria, which is a potent trigger for caspase activation (see below) [1046].

Some 'typical' initiation caspases may function as executor caspases in certain tissues. Such are, for example, caspases-1 and -11, which act as cytokine signalling activators in most tissues, but are capable of direct triggering of the apoptotic cascade in neurons and oligodendrocytes [1047-1049].

Some caspases have unique functions. Such is, for example, caspase-14, which plays a role in process of terminal differentiation of epidermal keratinocytes [1050,1051]. The apoptosis is not carried out in the typical manner in the epidermis, as the dying cells must to remain physically on-site and carry out most of their functions after they are already dead. In the mouse, caspase-14 is not cleaved by caspase-1, caspase-2, caspase-3, caspase-6, caspase-7, caspase-11 or granzyme B, but by murine caspase-8 only [1052]. It is also cleaved at an atypical site – namely, at an isoleucine (in man) or a leucine residue (in the mouse), which distinguishes it from other caspases that are uniformly cleaved at aspartate residues [1053].

The primary triggering signal for apoptosis may be induced by an external factor (exogenous) or may originate from within the cell (endogenous). The associated mechanisms of activation of apoptosis may be very different. The resulting apoptotic cascades converge at some point and usually the one pathway is augmented by the other. The triggering events in apoptosis are usually very specific, as it is very important that the signal for apoptosis could not be mistaken for another signal and could not be simply ignored (at least in normal (nontransformed) cells).

6.4.3. Receptor-mediated (exogenous) mechanism for activation of apoptosis

Receptor-mediated apoptosis is induced in the presence of a specific exogenous stimulus – typically, a 'death' ligand, binding to a designated 'death' membrane receptor. For example, cytotoxic lymphocytes usually employ the exogenous mechanism to trigger the apoptotic cascade in their target cells. Initially, the death ligand binds to the death receptor in the plasma membrane, then the ligand-receptor complex is internalised. Once inside the cell, it facilitates the recruitment of intracellular adapter molecules such as FAS-associated death domain protein (FADD). FADD (MORT1) is an adaptor molecule that recruits caspase-8 or caspase-10 to activated death receptors (Fas or TNFR-1) [1054]. It has a death effector domain, which facilitates binding of other DED-carrying proteins, such as caspase-8 [1055]. The adapter proteins recruit pro-caspase-8 molecules, which form an aggregate. Once the molecules in the aggregate are brought in vicinity of one another, they activate each other by partial proteolysis. Activated caspase-8 activates, in turn, its downstream proteins, including executor caspase-3 and other signalling and effector proteins.

6.4.4. Endogenous mechanism of activation of apoptosis

Activation of apoptosis by endogenous stimuli occurs when the cells are overstressed (e.g. oxidative stress) and their DNA has sustained serious damage. In response to severe damage, the outer membrane of mitochondria is permeabilised, which is the earliest event in the signalling cascade of the endogenous pathway. The direct apoptotic trigger, however, is the release of cytochrome c and other proteins, such as mitochondrion-associated apoptosis-inducing factor (AIFM1, see below) from the mitochondria. Cytochrome c is a heme-containing protein which is a component of the electron transport chain in oxidative phosphorylation [1056]. It is normally residing in the space between the two mitochondrial membranes, associated with the inner membrane. The released cytochrome c binds to the adapter protein APAF-1, the mammalian homologue of the pro-apoptotic ced-4 protein of C. elegans [1002,1057]. APAF-1 recruits pro-caspase-9 to form a ternary complex and induces its activation in the presence of dATP [1058]. Activated caspase-9 is capable of launching the downstream proteolytic cascade, eventually resulting in apoptosis [1035].

Mitochondrion associated apoptosis factor 1 (AIFM1) is a mitochondrial FAD-dependent oxidoreductase [1059]. In non-apoptotic cells it is attached to the mitochondrial membrane and acts as an anti-apoptotic factor [1060]. After the outer membrane of the mitochondrion has been permeabilised in response to pro-apoptotic signalling, AIFM is cleaved by partial proteolysis and is released as a soluble component (AIFM1sol) from mitochondria. AIFM1sol is then translocated to the nucleus, where it mediates the condensation of chromatin and degradation of DNA [1061]. Poly-(ADP-ribose)-polymerase-1 activation is necessary for the translocation of AIF from the mitochondria to the nucleus [1062]. AIFM1 is capable of induction of an alternative cell death pathway, resulting in programmed death of the cell even in the absence of activated effector caspases [1063]. Caspase-independent cell death is characterised by cell shrinkage, chromatin condensation without nuclear fragmentation (pyknotic cell death) and NAD+ and ATP depletion, related to PARP1 activation [1064].

Often, the endogenous pathway of activation of apoptosis is switched on in addition to the receptor-mediated pathway, in order to speed up the process and/or to aid in amplification

of the pro-apoptotic signal.

6.5. Relationships between DNA repair and apoptosis

As it was already discussed, acknowledging the presence of unrepaired damage in the DNA of a cell capable of division may constitute a major turning point in the cell's fate. If the damage is not very severe and/or extensive, repair activities are normally undertaken, including cell cycle arrest until the damage is successfully repaired, so as to avoid replication of damaged DNA. Different types of DNA damage may have radically different impact in the assessment about whether the cell will live or die. For example, the tolerance of the normal cell to double-stranded breaks is very low, as only a few of these may render the cell 'disposable' while a significant amount of thymine dimers in DNA may only result in temporary arrest of the cell cycle [1065]. If, after the repairs have been carried out, the cell's DNA has been re-checked and damage is still present, the cell is typically routed to the programmed cell death pathway (Fig. 21). Again, this may be viewed as a measure to avoid transmission of altered variants to the genetic pool.

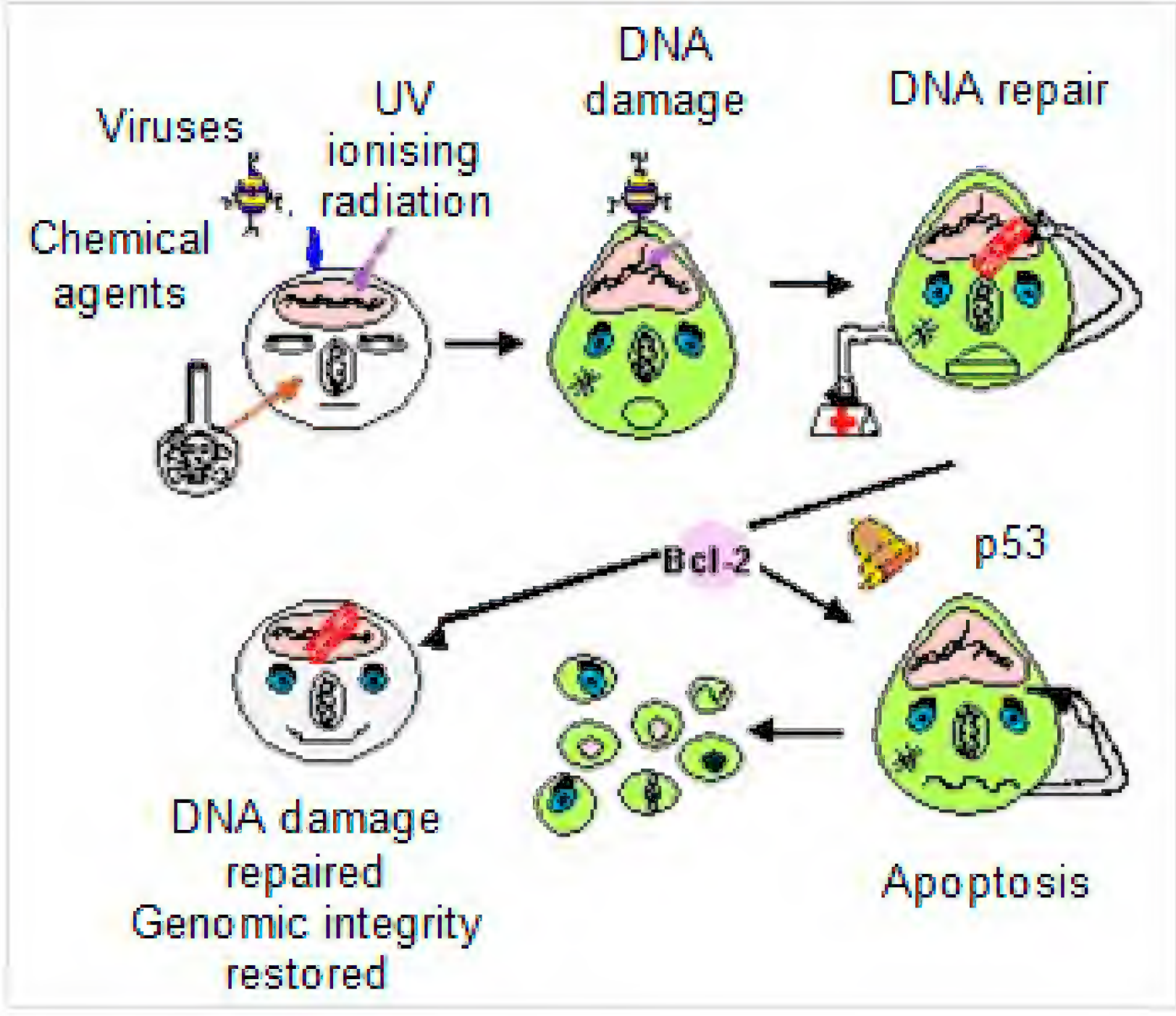

Figure 21. Repair/apoptosis relationships.

Usually, the persistence of unrepaired DNA damage in a normal (nontransformed) cell is a potent signal for activation of the apoptosis pathway. There are, however, exceptions to this rule. In specific cases (e.g. SOS response in bacteria, translesion replication in eukaryotes), the cell with damaged DNA may not be sacrificed, but may be allowed to live and, in some cases, reproduce. This is associated with risk for introduction of mutations during copying of damaged DNA templates.

Damage to mitochondrial DNA may initiate apoptosis independently of the state of the nuclear genome. This usually occurs by triggering generation of high levels of superoxide, which stimulates release of cytochrome c, and, eventually, apoptosis via the endogenous pathway [1066].

6.5.1. Role of p53-associated signalling in apoptosis

The true Master [of death] does not seek to run away from death. He accepts
that he must die, and understands that there are
far, far worse things in the living world than dying.
J. K. Rowling. Harry Potter and the Deathly Hallows (2007)

p53 plays a central role in the activation of apoptosis in damaged cells. As it was already mentioned, in non-stressed cells p53 is maintained at low levels by constant tagging for degradation by the E3 ubiquitin ligase MDM2 [712]. The activating signals (e.g. cellular stress, DNA damage) cause destabilisation of MDM2, resulting in release of p53 and its accumulation [716,733]. The activated p53 protein modulates the transcription of many downstream genes, containing in their regulatory sequences the p53 consensus binding element ([5'-XXXC-A/T) (T/A)GYYY-3']2) [683]. Different p53-response elements exhibit different affinity for p53, with some requiring only a small increase in the p53 level to be bound and activated and others while others will not be activated until reaching a certain threshold level [683]. A dose effect may be observed in outcomes of damage-related accumulation of p53. Levels below certain threshold generally cause cell cycle arrest and attempts to repair the damage, while very high (suprathreshold) levels may route the cell directly to the programmed cell death pathway [1067,1068]. Many genes coding for many pro-apoptotic proteins (e.g. *PIDD*(p53-induced protein with death domain), *NOXA*, *PERP*(p53 effector related to PMP22, a p53 target in apoptosis), *PIG8* (p53-induced gene 8), and others) contain a p53-binding element, which makes their expression directly dependent on p53 [1069-1071].

p53 may activate apoptosis by either the exogenous or the endogenous pathway, although in most cases p53-associated pro-apoptotic signalling results in induction of the endogenous mechanism of caspase acti¬vation [1071,1072].

Pro-apoptotic genes whose expression is directly stimulated by p53 may be broadly classed in three groups: genes coding for membrane proteins; genes coding for proteins of the cytosol; and genes coding for mitochondrial proteins. Major representatives of the first group (membrane proteins) are, for example, the proteins of the TNF receptor superfamily – CD95 (FAS), DR5 (death receptor 5, a TRAIL ligand receptor), PERP and others. They function in initiation of apoptosis via the exogenous pathway, activating the initiator caspases -8 and -10.

Among cytosolic p53-dependent pro-apoptotic proteins are, for example, PIDD and PIG8. They function in the activation of apoptosis via the endogenous pathway and may be directly activated by p53 [1073-1075]. In the presence of DNA damage p53 may activate the initiator caspase-2 via PIDD [1076,1077].

p53-controlled mitochondrial proteins with pro-apoptotic properties are, for example, the Bcl-2 related proteins Bax, Noxa, PUMA (p53-upregulated modulator of apoptosis) and others [1070,1078,1079].

p53 is capable of direct activation of transcription of genes coding for proteins that facilitate the release of cytochrome c from mitochondria [710,1080]. Proteins of this type are, for example, some of the proteins of the above mentioned Bcl-2 family. The latter comprises, besides the eponymous classic proto-oncogene, many other cytoplasmic proteins (Bid, Bim, Bax, Bak, Bad, Bok, Noxa, PUMA, etc.). Bcl-2 proteins are responsible for the maintenance of the integrity of the mitochondrial membrane in higher animals and man. Different Bcl-2 family members may exhibit pro-apoptotic or anti-apoptotic properties. For example, BCL2, an integral protein of the inner mitochondrial membrane, inhibits apoptosis by blocking the release of mitochondrial cytochrome **c** [1081]. Bcl-xL (BCLX) is also an anti-apoptotic protein, believed to promote cell survival by regulating the electrical and osmotic balance in the mitochondrion [1082]. Other members of the Bcl-2 family promote the initiation of apoptosis and/or amplify the pro-apoptotic signal (Bax, Bak1, and others. Anti-apoptotic Bcl-proteins share a high degree of homology with ced-9 protein of C. elegans [975].

6.5.2. Role of ATM/ATR in pro-apoptotic signalling

The ATM/ATR complex usually activates apoptotic pathways indirectly, by signalling to p53, which, in turn, activates the apoptotic machinery. In response to DNA damage (double-strand breaks), ATM may directly phosphorylate p53 at Ser15, causing its stabilisation and accumulation [749]. ATM may also phosphorylate and activate the checkpoint kinase CHK2, which, in turn, may phosphorylate p53 at Ser15 and additional serine residues (e.g. Ser20), resulting in increased p53 stability, accumulation and activation [733].

In the presence of unrepaired damage in DNA and/or unprotected free DNA ends (including chromosomes with telomeres shortened below a critical length), apoptosis may be induced by a p53-independent pathway [1083-1085]. p53-independent mechanism of apoptosis may involve ATM, checkpoint kinases, DNA-dependent protein kinase and the protein nibrin, product of the *NBN* gene [439,469,1086,1087]. Usually, the pro-apoptotic signal in the p53-indepemdent pathway is transmitted to BCL-2 family of proteins (specifically, BAX), causing release of cytochrome c from the mitochondrial membrane and activation of the endogenous pathway of apoptosis [1088,1089]. Nibrin may transmit the apoptotic signal to BCL-2 family proteins or directly to the executor caspase-3 [440].

ATM, but not ATR is a target of executor caspases in apoptosis [1090]. Usually ATM is cleaved by caspase-3, but caspase-7 is also capable of targeting ATM, albeit with lower efficiency [1091].

6.5.3. Role of other proteins of DNA repair in apoptosis

Detection of DNA damage and the transmission of the signal to p53 may also be carried out by the 9-1-1 complex instead of ATM/ATR-dependent signalling [248,649,1092]. 9-1-1-complex regulates two damage-dependent signalling pathways that eventually converge on p53 activation [784]. The 9-1-1 complex may activate p53 directly, on the one hand, or may induce cell cycle arrest by activation of checkpoint kinase CHK1 and inactivation of cyclin-dependent kinases until the final 'live or die' decision is made, on the other hand [870]. CHK1 is a downstream target of ATR and becomes activated upon phosphorylation. Activated CHK1 induces phosphorylation of MDMX at Ser367, eventually leading to p53 activation [721,1093].

Similarly to ATM, poly-(ADP-ribose) polymerase and DNA-dependent protein kinase are also direct substrates of executor caspases in apoptosis, leading to inactivation [1094]. PARP1 is among the first molecules of DNA repair that are inactivated in cells on their way to apoptosis. Active PARP1 transfers multiple ADP-ribosyl units onto various target substrates as well as its own molecule, using NAD+ as substrate. The catalytic action of PARP1 causes progressive decrease in NAD+ in the cell, which, in turn, results in depletion of ATP. Eventually, this may cause necrotic cell death. To avoid that, PARP1 is proteolytically degraded in pre-apoptotic cells by caspase-3 [902,903]. Accumulation of poly-ADP-ribosylated polymers in a cell is usually a potent signal for activation of the apoptosis programme [904]. Inactivation of PARP1 by caspases is viewed by some authors as an act equal to switching imminent death pathways from necrosis to another that is safer for the other cells (apoptosis) [1095,1096].

PARP1 may also mediate a caspase-independent pathway of programmed cell death in cells under stress (for example, cells in ischaemic regions damaged by hypoxia). Thus occurs probably by induction of translocation of soluble AIFM1 released from the permeabilised mitochondrial membrane to the nucleus [summarised in 1097 and 1098].

DNA-PK is a major target of executor caspases. Cleavage of DNA-PK by caspase-3 produces two fragments, one of which carries the catalytic domain of DNA-PK [1099]. This, however, is not sufficient to fully suppress the kinase activity. To overcome this, protein kinase C delta (PKC delta), which in its inactive state is a complexing partner of DNA-dependent protein kinase, is activated by caspase-3 during apoptosis. Activated PKC delta hydrolyses the fragment carrying the kinase domain of DNA-PK, inactivating it completely [1100].

The Ku subunits of DNA-PK play a role of their own in apoptosis. In unstressed cells, Ku70 binds to the pro-apoptotic protein BAX and suppresses its translocation from the cytosol to the mitochondria [1101]. Acetylation of Ku70 occurs early in apoptosis, causing dissociation of Ku70 from BAX and stimulating the pro-apoptotic properties of the latter [1102,1103].

Cyclin-dependent kinases may play a role in apoptosis as well. Apoptosis in certain types of cells (e.g. endothelial cells, cardiomyocytes, and some types of cancer cells) is associated with upregulation of cyclin A-associated CDK2 [841,1104,1105]. In apoptotic cells, the carboxy termini of the CDK inhibitors CDKN1A (p21, CiP) and CDKN1B (p27, KiP) are cleaved by caspase-3 [840,1106]. The affinity of truncated CDKN1A and CDKN1B to the cyclin-CDK2 complexes decreases, with CDKN1A leaving the nucleus altogether, resulting in increased cyclin A-CDK2 activity. Mutations in the *CDKN1A* gene conferring resistance to caspase cleavage may suppress apoptosis in human cells [840].

6.5.4. Biological role of caspases apart from programmed cell death

Caspases have additional biological roles except in programmed cell death (see Table 5). Cell differentiation is specifically dependent on caspase activity. This is, of course, in most cases related to the fact that differentiation of many cell types requires controlled destruction of cells that have not taken the correct differentiation route or have failed to comply with the requirements of a particular stage of differentiation.

The role of programmed cell death in differentiation is best studied in blood cells, as their differentiation programming normally includes 'default apoptosis' in case the cell has not received a specific 'survival' signal (e.g. transmitted through a growth factor or a cytokine

bound to a surface receptor). For example, several functional checks are carried out during differentiation of T-cells – for correct rearrangement of the genes coding for the chains of the T-cell receptor, for sensitivity to MHC-bound antigens and for reactivity of the T-receptor to self-antigens. All precursor cells that have failed to comply with any of these checks (that is, cells that have not completed the rearrangement of the T-cell receptor genes, have shown subthreshold affinity to antigens, or exhibit autoreactive properties) are routed to the suicide pathway by default. Only cells that have passed a check receive a 'survival' signal and may proceed to the next stage of differentiation, where many of them would again be assessed as noncompliant to the requirements of the stage and routed to apoptosis [1107,1108]. Only about 2% of all immature T-cells that have passed the checks for completion of rearrangement of T-cell receptor genes (double-positive ($CD4^+/CA8^+$) thymocytes) survive the antigen affinity check and the reactivity check. About 50 million new cells of the T-cell lineage are generated each day, but only about a million of these actually live to become mature T-cells, the remaining cells die by apoptosis [1109].

Differentiation of epithelial cells is also caspase-dependent, but involves a specific non-apoptotic caspase, namely, caspase-14. The cells of the stratum corneum of the skin must undergo specific changes in their metabolism and architecture before they become fully functional. What is most important, however, is that they are already dead before they are transported to the upper layers to perform their functions, but need to stay in place until they are sloughed off and not be 'eaten' by the surrounding cells, as normally happens with apoptotic cells. To achieve this, the autophagy of cell contents in epithelial cells does not proceed as usual, as the cell needs to remain essentially whole and certain proteins, such as keratins, are preserved [1110,1111]. The caspase enzyme responsible for epithelial cell differentiation (caspase-14) is expressed only in the cornifying epithelium of terrestrial mammals [1051]. It cleaves filaggrin, the main component of the keratohyalin granules in mammalian epidermis, thereby facilitating the keratinisation of differentiating epidermal cells [1112]. During differentiation of epidermal cells, the multimeric profilaggrin precursor is dephosphorylated and cleaved by serine proteases to form filaggrin monomers. The latter bind to the keratin fibrils in differentiating epidermal cells, aiding the process of cell compaction. Later, filaggrin is further modified and cleaved by caspase-14 into shorter fragments, then processed into hygroscopic amino acids, ensuring the moisture-retaining properties of the upper epidermal layers.

6.6. Disorders of induction of programmed cell death resulting in human disease

Even a broken clock is right twice a day.

Stephen Hunt, The Court of the Air (2007)

Disordered activation and inactivation of caspases and impaired interactions between caspases and adapter proteins may result in disease phenotypes in man. At the moment, genotype-phenotype interactions in human disease related to disordered initiation of caspase cascade, deployment of caspase activity and cleavage of target substrates are best studied in tumorigenesis and neurodegenerative diseases, but their role in other hereditary conditions is also under intensive study.

As caspases play very important roles in the life cycle of the cell, inherited conditions related to caspase deficiencies are relatively rare in man and mammalian models, with the

affected embryos probably dying early in utero. Partially caspase-deficient mouse and rat models are currently available, in which the caspase gene knockout is introduced only in a specific tissue. For example, mice with the caspase-8 gene inactivated or deleted in epidermal keratinocytes manifest with dry, scaly, thickened skin with significant degree of inflammation (Fig. 22) [1113]. This is believed to result from disordered IL-1α signalling and upregulation of caspase-1 [1114].

Figure ?. Mouse with conditionally knocked out *Casp8* gene in epidermal keratinocytes (below) and a normal age-matched mouse of the same strain (above) [1114].

Induced expression of caspase-8 in the skin of mouse models stimulates wound healing [1114].

In man, caspase-8 inactivation is believed to play a major role in the pathogenesis of skin changes in atopic dermatitis [1115].

Lab mice with T-cell – specific deletion of *Casp8* gene develop a severe lymphoproliferative T-cell disorder [1116]. Similar human lymphoproliferative conditions associated with caspase-8 and caspase-10 deficiency, respectively, have been described – autoimmune lymphoproliferative syndromes type IIA (ALPS2A) and type IIB (ALPS2B). The major feature of ALPS2A is loss of immunological tolerance to 'self' antigens, which may manifest as autoimmune cytopenia (e.g. anemia, thrombocytopenia), hepatosplenomegaly and lymphadenopathy [1117]. In ALPS2B additionally manifests with severe immunodeficiency with failure in activation of virtually all cells of cell-mediated immunity – B-, T-, and NK-cells, leading to recurrent infections [1118]. The related conditions of autoimmune lymphoproliferative syndromes type I A and B are also disorders of apoptosis. They are

caused by inherited defects in the *FAS* (CD95) gene and the FAS ligand gene (*FASL*), respectively [1119].

Transgenic mice with caspase-1 overexpression in the skin exhibit increased levels of apoptosis in epidermal keratinocytes and present with severe dermatitis and skin ulcers [1120].

No cases of human disease associated with disorders of caspase-14 expression and/or activity have been identified yet. Casp14 (-/-) knockout mice, however, were created several years ago [1051]. They were born at expected rates, generally healthy and fertile and with lifespan typical of wild-type mice. Their skin, however, was thick, shiny and hardened and prone to increased water loss. The skin of Casp14 deficient mice was very sensitive to formation of cyclobutane pyrimidine dimers after UV-B irradiation. This, however, was not associated with increased risk for skin cancer, but, rather, with increased rate of apoptosis of skin cells after UV irradiation.

It is believed that overstimulation of caspase activity is one of the pathogenetic factors in Alzheimer's disease [1121,1122]. Over-activation of caspase-3 and caspase-6 increases the rate of neuronal cell death in Alzheimer's disease, with caspase-3 playing a role in neuronal cell body apoptosis and caspase-6 in axonal degeneration [1123,1124]. A major histopathological hallmark of Alzheimer's disease is the presence of amyloid deposits (plaques) in the brain matter (senile plaques) and the blood vessels (cerebrovascular plaques) of the brains of affected patients [1125]. 'Amyloid' is a common term for a variety of fibrillar aggregates exhibiting β-sheet structure. Principal components of the amyloid plaques are the amyloid-β 1-42 peptide (in senile plaques, made of 42 amino acid residues) and the amyloid-β 1-40 peptide (in cerebrovascular plaques, 40 amino acid residues) [1126]. These are derived by partial proteolysis from the larger amyloid-β4A-precursor protein, consisting of 695 amino acid residues [1127]. Amyloid-β4A precursor was identified as a substrate of caspases -2, -3, and -8 [1123,1128]. Increased rates of cleavage of the amyloid-β4A precursor in Alzheimer's disease result in overproduction of its hydrolysis products, the amyloid-β 1-40 and amyloid-β 1-42 peptides, which accumulate in the brains of the affected individuals. Ischaemic and excitotoxic brain injury are believed to be among the major inducing factors for proteolysis of amyloid-β4A precursor [1121,1123]. Other neuronal proteins are also targets for caspase activity, such as beclin-1, a protein mediating the interactions with anti-apoptotic proteins of the *BCL2* gene family [1122,1129]. The levels of beclin-1 in brains of patients with severe Alzheimer's disease are drastically reduced, up to 30% of normal levels [1130].

Caspase-induced neurotoxicity is likely to play a role in the pathogenesis of other neurodegenerative diseases in man with suspected excitotoxic origin, such as Huntington's disease (HD). HD is a heritable monogenic disease with late onset, transmitted in an autosomal dominant fashion. The underlying defect is a trinucleotide ($(CAG)_n$) expansion at 4p16.3, resulting in occurrence of an abnormal polyglutamine stretch in the encoded protein (huntingtin). In Huntington's disease, there is a progressive loss of selected neuronal populations in the brain, especially in the striate nucleus and the cortex. This is also where the mutant huntingtin accumulates and forms aggregates. It is believed that proteins containing expanded polyglutamine stretches are cytotoxic in the nervous system, as about

10 inherited diseases in man (mainly neurological disorders, e.g. spinocerebellar ataxias) caused by abnormal polyglutamination have been identified so far. Glutamate receptor-mediated excitotoxicity is strongly suspected to contribute to neuronal loss in HD [1131]. Mutant huntingtin was shown to activate caspase-9 and caspase-3 and to stimulate the release of cytochrome c from mitochondria [1132,1133].

APAF1 expression is often lost in metastatic melanoma [1134,1135]. Genomic rearrangements at the 12q locus in man, producing inactivation or loss of the *APAF1* gene are a predictor for poorer outcomes in cutaneous melanoma [1136]. In some melanomas, the APAF1 expression may be restored by various chemotherapeutics – e.g. demethylating agents (5-aza-2-deoxycytidine). As loss of *TP53* gene copies is not typical for melanoma, the programmed cell death pathway may be reactivated in tumour cells that were previously incapable of apoptosis, provided that they have not lost their *APAF1* gene copies altogether.

Dark, the Drosophila homologue of APAF1 adapter protein, was shown to play a role in pathological polyglutamine aggregation, associated with abnormal activation of programmed cell death [1137]. Inactivation of Dark was found to suppress the formation of polyglutamine aggregates in Drosophila.

Inborn defects in the human *AIFM1* gene (Xq26.1), encoding a mitochondrial protein with oxidoreductase activity capable of activating caspase-independent apoptosis, may result in Cowchock syndrome (Charcot-Marie-Tooth disease-4) [1138] or combined oxidative phosphorylation deficiency 6, a mitochondrial disease associated with early-onset progressive neurodegeneration [1061].

A rare type of autosomal recessive mental retardation – type 34 (MRT34) is believed to be caused by homozygous mutation in the *CRADD* adapter gene (12q22) [1139].

Molecular defects affecting the expression of the pro-apoptotic protein BCL-2 (overexpression, ectopic expression) play a role in the pathogenesis of some haematological cancers – follicular lymphoma, chronic promyelocytic leukemia, etc. In follicular lymphoma, a translocation (14;18) often is found, moving parts of the *BCL-2* locus (normally on chromosome 18) to the *IGH* locus on chromosome 14, where the genes coding for the heavy immunoglobulin chains are located [1140]. This is believed to result from errors in joining of free DNA ends in V(D)J recombination at early stages of B-cell differentiation [1141]. In variant follicular lymphoma, the 5'-part of the *BCL-2* locus is transferred to chromosome 2, close to the locus of the kappa light immunoglobulin chains [1142]. In chronic lymphocytic leukemia, a (18;22) translocation is often found, moving the 5'- portion of the *BCL-2* gene from chromosome 18 to chromosome 22, close to the loci containing the genes coding for the lambda light immunoglobulin chains [1143]. The translocated *BCL-2* proto-oncogene is usually constitutively expressed, inhibiting the normal 'default' mechanism of apoptosis in differentiating B-cell precursors that have failed to comply with requirements at a certain stage of differentiation (IgM-secreting cell for chronic B-lymphocyte leukemia, mature B-memory cell for follicular lymphoma), resulting in haematological neoplasia [1144-1146].

6.7. Programmed cell death as an adaptive mechanism

Logic clearly dictates that the needs of the many
outweigh the needs of the few, or the one.

Star Trek II – The Wrath of Khan (1982)

Programmed cell death (apoptosis) serves to remove damaged, infected, or transformed cells from the cell population without damaging the neighbouring cells and without triggering immune defences and/or tissue regeneration. The decision whether to engage the programmed cell death routine is typically made before DNA is replicated, as a precaution against propagation of cells carrying DNA that is different from the original blueprint. Special care is taken that the cell's DNA is made unavailable and the tools normally used to repair it are properly destroyed before the cell actually dies.

It has only recently been proposed that the maximal lifespan of the individual and their death are integral part of their inbuilt genetic programme, similarly to individual development [1147]. Indeed, not that long ago, it was postulated that after the stage of active growth of the organism has been completed and sexual maturity has been achieved, the role of the genetic component was effectively over. Thus, the further destiny of the ageing adult organism was assumed to be determined by constellations of random events of exogenous as well as of endogenous origin, producing natural wear and tear of tissues and organs, some or all of which would eventually cause the death of the organism. At present, it is believed that not only the early stages of development follow a preprogrammed course (in stages) but also adult life, old age and even death. At each of these stages the default programme may be delayed, diverted to an alternative pathway, or possibly even reset (purely hypothetically), but it cannot be altogether avoided or permanently halted.

Several large gene families involved in the implementation of the individual developmental programme have been identified so far. Their working principle is based on ordered switching on and off of the genes required for the successful completion of every phase of development. Among these are, for example, the homeotic gene clusters, in which the separate genes are activated and deactivated in an ordered fashion; or the genes that implement the epigenetic activation or inactivation of other genes (e.g. the *MECP2* gene in man) during the individual development of the organism from the conception to adulthood. Similar pathways and mechanisms may be responsible for the ordered implementation of the late-life individual programme and possibly the death of the organism. Programmed death of whole organisms is, after all, completely possible and is legitimate part of the life cycle in some organisms (e.g. annual plants, ephemeral insects, etc.). This death pathway is independent of external factors (availability of water, food, temperature, etc.) What is more, at the point of death the cells and tissues of these organisms are, for all intents and purposes, 'fit' for living beyond the point of completion of death programme.

Ageing and 'dying of old age' are currently often viewed as the equivalent of apoptosis, but on higher-order level (tissues, organs and system, organism). According to this theory, ageing and death of old age exist to make sure that no living thing (except some very simple organisms) lives longer than its allotted time, as very long-lived creatures might present a danger to the population's genetic pool, Essentially, it is the same type of danger that damaged cell that has abolished the control mechanisms ordering it to die would be to the other cells in the tissue. One could hypothesise that the mechanisms of ageing and death of old age have evolved in order to ensure effective, timely and complete 'extraction' of

damaged DNA from the genetic pool. These two mechanisms may be viewed as equivalents of cell cycle arrest in damaged cells (ageing) and programmed cell death (death of old age), on a population and supra-population scale. They make sure that the genetic diversity of the population and the genetic integrity of the species would not be lost because of incidental predominance of a limited number of mutant genetic variants conferring immortality to their carriers.

If all other attempts to remove the carrier of the damaged or altered DNA (be it a single cell or a sophisticated living being) from the genetic pool fail, the result is cancer. All modern anticancer treatments eventually fail and cancer that cannot be radically cured at early stage may be slowed down, but it finally kills the organism it originates from. It is likely that no 'universal' cure for old age, death of old age and cancer would ever be invented, as these are Nature's own safeguards against immortality, and man has scored but little victories in battles against Nature's laws so far. This is no reason for despair, however, as modern science and medicine invent virtually every day more and more diverse ways for humans to live comfortable the time they naturally have, instead of chasing the impossible dream of making them live forever.

For more information on the modern theories of ageing, death of old age and cancer.

7. DNA repair and ageing

I wasted time, and now doth time waste me.

William Shakespeare, Richard II (1595). Act V, scene 5.

7.1. Cellular senescence and ageing of multicellular organisms

Ageing is a process that occurs in virtually all living things (starting from single-cells and ending with complex organisms), and ends in death of the cell or the organism. There is no universal definition of ageing, as there are no universal definitions of life and death. Usually, ageing is defined by descriptions of the traits that may be associated with it, but these traits are not universal to all types of ageing or to all ageing creatures.

Generally, the term 'ageing', when referring to a cell, is associated with specific defined properties (e.g. permanent replicative arrest), but these may not be valid for all types of cells. For example, terminally differentiated cells may exist for a very long time in G0 and never actually get to divide again, but this reflects their extreme functional specialisation and is typically not associated with inevitable death of the cell in the near future. What is more, it is known that some nontransformed cells that are normally quiescent may re-enter the replicative cycle, should the need arise. Hepatocytes are the most popular example. They are specialised cells that are usually in G0, but are fully capable of division and may, in case the liver has been damaged, completely restore the bulk and the functionality of the organ.

In a multicellular organism, 'ageing' refers to the process in which the internal reserves of the organism for combating disease and all types of stress gradually decrease. The capacity of the organism for adaptation to changing environmental conditions may also decline. The mortality rate, which is usually low in younger organisms of the same species, usually rises

as the organisms age. This, however, has its exceptions – for example, the major peaks in mortality for some species of stony corals occur in the juvenile (larval) stages [1148].

Until recently, it was actually believed that prokaryotes did not experience ageing as such and that the viability of bacterial cells was entirely dependent on environmental conditions. About a decade ago, it was demonstrated that a process resembling the essential features of ageing could actually take place in bacteria and oxidative stress was proven to play a major role in it [1149,1150]. Specifically, they found that as the growing E. coli population reaches stationary phase, the distribution of cell components in dividing E. coli may become asymmetric, with one of the daughter cells receiving the 'old' proteins of the mother cell (presumably carrying oxidative damage) and the other daughter cell receiving only the newly synthesised ones. Those cells that inherited the 'old' components experienced gradual but irreversible decrease in growth rate and eventually died [1151]. Simple unicellular eukaryotes (e.g. some species of yeast) generally exhibit finite replicative capacity and ageing may occurs in yeast – indeed, after different number of divisions that makes for variable time intervals [1152,1153]. Most multicellular organisms, however, begin to age progressively and eventually die within relatively broad time limits, varying from months to years (depending on the average lifespan of the species).

There are some notable exceptions to the rule that all living things age and eventually die. For example, in some very simple multicellular organisms replicative ageing never actually occurs, so they may live for a very long time and may be considered practically immortal ('immortality' in this case means only that the cells and the organisms cannot die 'of old age'). Among these are some of the members of the phylum Cnidaria – for example, members of the genus Hydra and a jellyfish with a rather peculiar life cycle called Turritopsis dohrnii (for details, see below). To be precise, in Hydra there is a process that may distantly resemble ageing (gradual decline in capacity for motion, capturing food and reproduction, and eventually dying), but it is not obligate and may not occur, as it is linked with sexual differentiation. Whether the later would occur, depends on the environmental conditions (e.g. availability of food). When food is plentiful, hydras are capable of continuous asexual reproduction by budding [1154]. In adverse environmental conditions, hydras revert to sexual reproduction, with the fertilised eggs settling on the bottom of the pool to be hatched when the environmental conditions have improved, and the parent hydras ageing and dying. Thus, the progeny that is left behind carries a genetic mix somewhat different from the genetic background of the parents that would hopefully help them survive the current environmental adversities.

Apart from the rare examples seen among Cnidarians, death is imminent for virtually all living creatures that have reached or exceeded the average lifespan for the species. The exact course of the process of ageing and when and how death would occur, however, are strongly individual and prognostication is usually unreliable, unless within very broad limits. For example, all humans on Earth belong to the same species – Homo sapiens, but there are serious variations between the average life expectancy from country to country, from population to population and even from man to man, even between people in the same family, and between individuals with the same disease or condition. Of course, there is the question of the quality and the availability of healthcare in different countries and to

different groups of people, as the life expectancy may vary up to decades depending on the access to healthcare and the quality of health services. For example, in 1841, when the Office for National Statistics of UK started their records of life expectancy, it was on the average 45 years for men and 49 for women, respectively. One hundred and seventy years later, in 2011, the average life expectancy in United Kingdom was 79 years for the men and 82 years for the women (according to WHO data for 2011). This formidable growth of average life expectancy of roughly 70% is not only result from better living and working conditions and from generally healthier lifestyles of people of today, but from much better and more accessible healthcare than it was in middle XIX century.

In other EU countries, the contribution of improvements of healthcare may be less obvious, but still significant. For example, according to the data published by the Bulgarian National Centre of Public Health and Analyses the average life expectancy in Bulgarian in 1935, when the records first started, was about 51 years for the men and 53 for the women. By 2012, it was about 71 years for the men and 78 years for the women, which make for a growth of 40 – 45% in less than 80 years.

On a global scale, the average life expectancy is highest in advanced non-European countries, such as Japan, where, on the average, the men live 79 years and the women live 86 years. The latter is a curious finding, as it is believed that the late consequences of the Hiroshima and Nagasaki bombing in 1945 would continue having impact on the health and the life expectancy of people of Japan for years to come from now. However, the first officially recognised survivor of the two atomic bombings in 1945 – Tsutomi Yamaguchi – actually died in 2010, 65 years after the bombings. Indeed, Yamaguchi died of cancer, but he was 93 years old at the time of death. The oldest living person (almost 116 years by the end of 2013) is currently Misao Okawa, a Japanese woman, and the former title holder was also Japanese – Jiroemon Kimura (died at 116 years and 54 days).

Life expectancy in man has apparently risen in the last 150 years, but this was largely because of improvements in control of infectious disease (e.g. introduction of antibiotics) and decline in perinatal and child mortality. Indeed, modern medicine has invented efficient ways to prevent or delay some of the adverse outcomes of 'diseases of old age', and the number of people aged >85 increases in all modern societies, but the oldest old of the present do not actually live longer than the oldest old of the past have lived. There have always been reports of people living to 90 – 100 years and beyond throughout human history. Therefore, modern science and medicine have not discovered ways to make life longer yet. Rather, they allow people to stay healthy until a more advanced age than before and provide options for people that are affected with diseases which were considered virtually untreatable or were offered palliative treatments only several decades ago. For example, while the average life expectancy is the longest in the Japanese, at the same time statistics shows that the prevalence of colorectal cancer has increased nearly 3 times compared to the prevalence recorded in the 40-ties of the XX century [1155,1156].

Below we will review the major points of what is currently known about the process of ageing – its origins, the mechanisms driving the process of ageing, and the factors that may play a role in the course of ageing.

7.2. Historical review of theories of the origins of ageing

Why do we age? The possible causes of the phenomenon of ageing have been a focus of interest throughout the history of science and medicine and have been investigated very thoroughly in the last several decades. Perhaps the first theory trying to explain the phenomenon of ageing was the 'wear and tear' theory of the German biologist August Weismann (1834–1914). He proposed that ageing of cells, tissues and organs in the human body was a product of overuse and abuse. Of course, there was no way around the fact that people that did not 'overuse' their body would also age, but it was assumed that people who did would age faster.

Later came the 'waste accumulation' theory that postulated that cells accumulate waste products that would, in time, kill them. Accordingly, a number of 'cleansing' procedures were recommended to help clean out the waste. The Russian biologist Ilya Mechnikov (1845–1916) and, after his death, the British surgeon Sir Arbutnot Lane (1856–1943) were ardent advocates of the theory that accumulated waste (particularly, in the colon) could make people ill (alimentary toxemia) and shorten their lives. They recommended regular 'cleansing procedures' (specifically, enemas) and even colectomy for a variety of diseases, including schizophrenia and manic depression [reviewed in 1157 and 1158]. Unfortunately, even in our day there are still people that practise 'cleansing' procedures (e.g. colon cleansing, various herbal remedies, etc.) as anti-ageing measures.

Around the first decades of the XX century emerged the 'incorrect reconstruction theory', proposing that in ageing individuals the body produced increasingly inadequate or incorrect 'reconstruction materials' and could not regenerate itself properly. This also spawned a following – for example, Paul Niehans's 'fresh cell therapy' that started being practiced in the 30-ties of the XX century [1159]. 'Fresh cell therapy' promised restored health and rejuvenation by introducing 'fresh' cells within the body of the patient, that could presumably repair his damaged tissues and organs. Notably, these cells were not even of human origin. It is truly astounding how 'fresh cell therapy' may be actually flourishing even today, after the repeated reports of deaths following Niehans-type of rejuvenation treatments with animal (usually, sheep) cells [1160-1162].

Examples of other rather short-lived theories of ageing are the 'multiple hormone deficiency' theory, the 'immune suppression theory' and the 'loss of cellular water' theory. The names are quite self-explanatory.

It was the 'free radical theory' proposed by the American biochemist Denham Harman in 1956 that really laid the bases of modern theories of ageing [1163]. According to Harman, the main reason of ageing of cells and tissues was damage of cell components by endogenously generated influx of free radical species. Harman's theory did not find much support at the time it was first formulated, and only became focus of interest after the mid-60-ties, when the idea that inherited defects causing accumulation of unrepaired damage in DNA could actually cause disease was unequivocally demonstrated and the initial premises of the 'molecular clock' theory of ageing (see below) were already in place [1164]. Later, in the 70-ties, Harman was one of the supporters of the 'mitochondrial damage' theory of ageing that proposed that free radicals generated by energy conversion in living cells caused oxidative damage in the mitochondria, which effectively triggered the ageing process

[1165]. The theory of mitochondrial damage contributed to the modern theory of ageing as a process triggered by accumulation of DNA damage.

Nowadays, ageing is viewed as a genetically pre-programmed process and not a product of random wear and tear of tissues and organs or accumulation of waste products. There is no unified theory of ageing yet. It is generally believed that ageing arose as a natural adaptive mechanism, targeted at preservation of the structure of populations and the genetic diversity in the course of evolution [1147,1166].

7.3. Current opinions about origins of ageing – 'the molecular clock theory' and accumulation of unrepaired DNA damage

Time is a dressmaker, specialising in alterations.

Faith Baldwin (1893–1978)

The 'molecular clock' theory

The 'molecular clock theory' has always been (and still is) one of the most popular theories about ageing. There is plenty of experimental data supporting it, and, of course, there are observations which indicate that it may not be always valid. Briefly, this theory postulates that the maximal lifespan of cells in multicellular organisms and the organisms themselves is contained in their genetic makeup as the maximal number of cell division cycles. Upon reaching the allotted limit of divisions the cell enters replicative senescence and eventually dies. Thus, the remaining time of life for the organism is measured by the remaining number of cell divisions.

The number of the divisions that a cell could manage before succumbing to replicative senescence is often termed 'Hayflick's limit', after the name of the American biologist Leonard Hayflick (1928). In the beginning of the 60-ties of the XX century, Leonard Hayflick and his co-worker Paul Moorhead experimented with primary cultures of human cells. They noted that the cells in the culture divided a finite number of times, then died. Further observations showed that the cells divided at a steady rate until approximately 50 population doublings, then began to decline. The closer the cultured cells were to the limit of 50 divisions, the more traits associated with ageing they exhibited [1167].

The number of cycles that a cell is capable before reaching the Hayflick's limit may greatly vary in different types of cells and between different species. For mammalian cells it is usually between 20 and 100 population doublings, but in short-lived species it is usually closer to the lower limit while in long-lived species it may reach the upper limit of the range. For example, cells from mice (rarely living beyond 2–3 years) divide only about 15 times before entering replicative senescence [1168], while cells from giant turtles that often live beyond 100 years may divide up to 110 times in culture [1169]. Hayflick's limit in different types of normal human cells may slightly vary, but the average is about 52 divisions. Cultured human cells from patients with progeroid syndromes (total progeria, Hutchinson-Gilford syndrome, as well as segmental progeria, e.g. Werner syndrome) are only capable of about 20 divisions before entering replicative senescence [448,1170].

It is theorised that 'resetting' or 'adjusting' the molecular clock may prolong the life of individuals with progeroid syndromes. It has been already demonstrated that upregulation of telomerase activity in cultured cells of patients with Werner syndrome may increase the

number of cell divisions before replicative senescence occurs almost up to the Hayflick's limit for normal cells [1171].

There is a direct relationship between the limit of Hayflick and telomere length. Specifically, Hayflick's limit usually coincides with the critical telomere length beyond which replicative senescence occurs [1172,1173]. Usually, the cells in the early embryo exhibit considerable telomerase activity. Beyond the embryonic stage, the enzyme activity that restores the length of the telomeres of somatic cells is usually undetectably low, so all somatic cells in the multicellular organism would enter replicative senescence and die in their own time, bringing about the end of the organism. In adult organisms, however, virtually every tissue (even brain tissue, which was until not long ago believed to be completely incapable of regeneration) maintains its own modest stock of cells whose primary function is to replace the lost somatic cells of the tissue. These are called adult stem cells.

Stem cells in adult tissues are well protected from possible sources of genotoxic stress. They are usually well insulated from the blood vessel network in order to be kept in slightly hypoxic conditions, their metabolism is turned down to the bare minimum, and most of the time they are in a state of replicative quiescence, as every cycle of division is associated with risk of introduction of replicative errors in the original blueprint. Some types of adult stem cells may retain their telomerase activity (e.g. haematopoietic stem cells, gamete-producing cells), albeit not as much as embryonic cells. Other types of cells in the adult body may possess the ability to re-activate the telomerase activity – for example, during liver regeneration after partial hepatectomy [1174].

As the organism ages, the replicative potential of adult stem cells also declines, therefore, the supply of new cell to make up for those that were lost slowly decreases in the ageing organism [1175]. This may manifest as degenerative disease. The decrease in the regenerative ability in advanced age is not always associated with rapid decline. Rather, the rate of regenerative tissue repair slows down and settles at a pace optimal for the aged organism.

Immortal (capable of unlimited division) cells are, for example, cancer cells, pluripotent stem cells (e.g. embryonic stem cells) and some immortalised (stabilised) cell lines. The length of telomeres in cancer cells may greatly vary – from very long (e.g. the CCRF-CEM cell line) to very short (e.g. in T-prolymphocytic leukemia). Cancer cells may up-regulate the activity of telomerase to maintain the length of their telomeres or may use alternative mechanisms for telomere maintenance.

Accumulation of unrepaired DNA damage

At present, it is believed that the ageing process is triggered by accumulation of unrepaired damage in DNA of eukaryotic cells. There is much of simple logic to this theory, and there is plenty of experimental data to support it. Briefly, the longer the life of a cell (or organism, for that matter), the greater the risk for occurrence of damage. Since DNA repair mechanism are not 100% error-proof, the more the damage, the higher the risk that some instances of damage may be 'missed' by the repair mechanisms. DNA damage that still persists after all the checks put in place to ensure the genome integrity before the onset of replication may cause copying errors that becomes fixed in the newly synthesised DNA and transmitted to the progeny. The mutation burden, therefore, gradually increases as age

progresses and the efficiency of DNA repair mechanisms declines. Eukaryotic cells could tolerate only so much mutations per genome, and cells that have experienced too much mutation 'hits' become incapable of further division (that is, they enter replicative senescence). This goes not only for somatic cells, but also the cells of the stem cell niche, therefore, the supply of new cells to make for those that got injured or lost gradually decreases, which manifests as typical age-related changes – e.g. atrophy of muscle, fat and connective tissue as well as degenerative 'diseases of old age' – atherosclerosis, cardiovascular disease, etc. One specific type of DNA damage – oxidative damage – allegedly plays a crucial role in ageing. As oxidation of substrates is the main mechanism for generation of energy in living cells, life is impossible without oxidative phosphorylation. At the same time, it is the main source of free radical species (mainly reactive oxygen species, ROS) in the cell. ROS cause oxidation of nitrogenous bases in DNA and introduction of double-strand breaks. Depending on how severe is the oxidative damage in the cell; it may be repaired by the designated mechanisms of BER and double-strand break repair and/or may trigger cell death by necrosis or apoptosis. It is experimentally proven that the amount of unrepaired oxidative damage in DNA increases with age – for example, the rate of occurrence of unrepaired 8-oxoguanines and double-strand breaks in mouse cells increases with age [1176]. With ageing, the levels of some of the enzymes in the brain which act in the detoxification of oxidised substrates, such as superoxide dismutase (SOD), catalase, glutathione transferase, and others gradually decline. This may be observed in normal ageing, but in brains of patients with Alzheimer's disease it may begin earlier and may follow a more rapid course [1177]. This may constitute research evidence supporting the concept of accumulation of oxidative damage as a major factor in the pathogenesis of Alzheimer's disease and Alzheimer-like states.

As mutagenesis occurs at random, the higher the mutation rate, the higher the risk that a mutation would occur in a gene that is directly or indirectly linked to regulation of the progression through the cell cycle, which may trigger neoplastic transformation of the affected cell/s. Unrepaired DNA damage is believed to be chiefly responsible for carcinogenesis in late life. The prevalence of almost all types of cancer increases with age. On the one hand, this may be because the aged organism has been exposed to genotoxic influences for a long time. On the other hand, the longer the individual lives, the higher becomes the risk that the events resulting from persistent genotoxic damage may trigger cancer growth. However, cancer is not always implicitly associated with ageing. For example, some of the segmental progeroid syndromes, such as Werner and Bloom syndrome, are associated with increased incidence of various tumours. In other types of segmental progeria, however, such as Cockayne syndrome, cancer is not usually part of the clinical presentation. Cancer-proneness is not typical for total progeria also (syndrome of Hutchinson-Gilford) as well. This may be, however, because of the severely shortened lifespan of those affected with Cockayne and Hutchinson-Gilford syndrome, and it is currently unclear whether affected individuals would eventually develop tumours if medicine could find a way to prolong their lives. As of now, however, it is considered that increased levels of unrepaired DNA damage are not sufficient to produce cancer – unless, of

course, it is a hereditary cancer associated with carriership of one defective copy of a specific gene (tumour growth being unleashed via the double-hit mechanism).

Carcinogenesis requires years and sometimes decades, because of the random nature of mutagenesis and the presence of efficient mechanisms for repair of DNA damage and/or elimination of damaged cells from the pool. Time, however, is not an independent factor in carcinogenesis, as it is known that cancer prevalence increases with age, but not in a linear fashion. Studies of cancer prevalence among the elderly (>75 years of age) show that in the age group over 90 the prevalence of cancer is lower than in those before 90 [1178-1180]. More specifically, these authors found prevalence of cancer of 35% in the group of elderly individuals aged 75–79; 20% in those over 90, and 15% among centenarians. One could speculate that the majority of these 'oldest old' may be free of cancer exactly because they had a very efficient DNA repair mechanism, allowing them to promptly repair genotoxic damage before enough errors have accumulated so as to trigger tumorigenesis.

7.4. We didn't have this in my day: the emergence of ageing-related and chronic diseases

Middle age is when your age starts to show around your middle.

Bob Hope (1903–2003)

As humans of today live longer and their living and working conditions improve, age-related diseases and conditions that were seldom observed in the past rapidly became the most commonly seen human diseases of our day. Common examples are atherosclerosis, cardiovascular disease, diabetes type 2, various cancers, senile cataract, macular degeneration, Alzheimer-type and vascular dementia, and others. Many of these diseases cannot be cured even now, but the affected individuals may have better quality of life and have normal or near-normal life expectancy. The potential impact of age-related disease on the healthcare system was predicted several decades ago, but its extent has only begun to be appreciated. A report by the Royal College of Physicians from September 2012 states that 65% of people admitted to hospital are ≥ 65 years old; with people over 65 accounting for 70% of bed days and people over 85 accounting for 25% of bed days at any one time [Hospitals on the Edge]. It could be expected that the situation of today is actually just 'the tip of the iceberg' and that the prevalence of diseases of ageing would continue to increase in the following decades.

The prevalence of another type of diseases has also increased lately, namely, those that were very rarely seen in the past because they arose as chronic consequences of pre-existing acute conditions that typically caused the death of the patient. With the arsenal of modern medicine, the affected individuals of today usually survive the initial acute attack. Many of them, however, are at increased risk for development of associated diseases or conditions. The latter usually develop some time after the onset of primary disease (from weeks and months to years); may be severe (sometimes life-threatening) and/or follow a chronic course. Examples are numerous. For instance, myelodysplastic syndromes (MDS) often precede by years (sometimes by decades) the development of overt leukemia [1181,1182]. MDS by itself may cause potentially life-threatening symptoms (anemia, thrombocytopenia, agranulocytopenia, etc.). Before the advent of antibiotics, genotoxic chemotherapeutics and the haematopoietic growth factors such as erythropoietin and G-CSF MDS often brought the death of the patient because of susceptibility to infections,

profuse bleeding, etc. Post-MDS leukemia was, therefore, quite rare, as only a proportion of the patients survived for long enough to eventually progress to full-blown leukemia. Today, however, the prevalence of people living with MDS is quite high (with about over fifty thousand new cases diagnosed every year), and the proportion of the so-called 'high-risk' patients in which MDS may transition to acute leukemia is higher than it used to be.

Similarly, post-streptococcal glomerulonephritis with subsequent renal failure and post-streptococcal rheumatic endocarditis are common late complications of infection with beta-haemolytic streptococcus even in our day, but only a century ago it was much more likely for the patient to die from the infection itself than from its late sequelae. Finally, diabetic ketoacidosis may be life-threatening even today; therefore, in the times when etiological treatment was not available, hyperglycaemia was much more likely to kill the affected person in acute settings, before the late complications (e.g. vascular disease, neuropathy, cataract, etc.) could develop.

To sum it up, today we experience a drastic increase in the prevalence of two types of diseases that were rare or very rare in the past – the diseases 'of old age' (chronic degenerative disease and cancer) and the diseases that may develop following an acute condition that used to be fatal but is now manageable in most cases. It could be easily (and wrongly) assumed that some (if not all) of these newly emerging diseases are a product of the modern lifestyle and/or environmental factors that were not present or were relatively rare decades and centuries ago. For example, many of the features of the metabolic syndrome and its late complication diabetes type 2 (e.g. central obesity, hypertension, elevated total and LDL cholesterol levels) may be attributed to overindulgence and lack of physical activity. Indeed, any and all of these three symptoms may result from unhealthy lifestyle (unhealthy eating patterns, lack of exercise, etc.) It is well known, however, that obesity, hypertension and hypercholesterolemia tend to run in families. One could argue that members of a nuclear family share a common environment and may, therefore, have the same unhealthy habits. Then again, very similar features may be seen in members of extended families that have never seen each other in their lives and have very different lifestyles. Clearly, lifestyle and habits may account only partially for the emergence of 'modern diseases'.

It is known now that the predisposition to develop a certain disease or condition may be heritable, but the associated condition may or may not develop. Whether it would actually develop, at what age and how severe its course would be depends on many other factors, genetic as well as environmental. For example, glucose intolerance is more common in children of parents with diabetes type 2 than in children of non-diabetic parents, and the risk for developing glucose intolerance is higher in individuals who had both parents with diabetes type 2 than in these that had only one diabetic parent. Adequate lifestyle modification (e.g. maintaining healthy weight, diet low in carbohydrates and fats, regular exercise. etc.) and regular check-ups for latent glucose intolerance and hypertension may delay the onset of diabetes or even prevent it altogether. This is valid, however, for virtually all individuals, regardless whether they have or have not family history for diabetes.

The risk for development of multifactorial diseases and conditions often increases as age progresses. For example, the risk of cancer and degenerative disease is usually higher in

older individuals (except in the oldest old, in which the incidence of cancer may actually be lower than in the 'younger old'). For other diseases and conditions with multifactorial genesis, the risk may be higher within a certain 'window' in the timeline of the individual, then decrease to the general risk for the population. An example is multiple sclerosis (MS), a multifactorial disease with strong genetic component. The development of MS is usually triggered in susceptible individuals by factors of the environment (viral infections, stress, etc.). The risk for MS is higher in the ages between 20 and 50, especially for women (male/female risk ratio is about 2.5), then gradually declines up to the age of 60, when the risk becomes low for both sexes.

Many human diseases and conditions are clearly heritable, according to data gathered by family and twin studies, but their development is also dependent on other factors, among which the environmental component may or may not play a role. These diseases and conditions exhibit high prevalence in all populations and yet it is very hard to assess the risk for the disorder for the particular individual. Such are, for example, autistic spectrum disorders (depending on the diagnostic criteria the reported prevalence may vary between 0.1% and >1%); schizophrenia (about 1%) and affective disorders (between 10 and 20%) in all populations [1183,1184]. Individuals from families with one or more affected members are at increased risk for developing the familial disease or condition but the risk if never 100%, even in identical twins. For the three conditions listed above, the risk for one member of an identical twin dyad to have the condition if the other identical twin has it is, respectively, 60–90% for autistic spectrum disorders, 50–60% for schizophrenia, 40–50% for bipolar I disorder and about 75% for the broad spectrum of affective disorders [1185-1187]. Reported concordance rates for dizygotic twins are much lower, typically similar to the rates of concordance between siblings. Apparently, having inherited the same genetic background that caused development of disease in other individuals is not enough to always cause the same disease or condition. It has been proposed that some of the common mental diseases and conditions may be related to receiving too large doses of alleles which, in smaller doses, may confer some kind of positive advantage [1188]. Thus, the allelic variants that in larger dosages were presumably associated with development of mental disease were actually selected for during evolution, with the less successful combinations eliminated from the genetic pool in every generation. This theory is very similar to the explanation of the high prevalence of globin gene variants associated with thalassemia or sickle cell anemia in regions where malaria is endemic. According to the latter theory, heterozygous carriers of some globin alleles were at significant advantage over non carriers, being resistant to malaria. Until recently, homozygous carriers of defective globin gene copies were likely to die early because of the associated genetic disease, therefore, their contribution to the genetic pool has been negligible throughout the course of human evolution. Thus, the high prevalence of alleles associated with severe disease when in homozygous state was maintained in the population because of the increased 'fitness' of heterozygotes. So far, none of the putative selective advantages of smaller dosages of alleles associated with increased risk for development of mental disease has been identified.

It is now theorised that the impact of individual genetic background on health and potential for longevity may be different in different ages. Specifically, some genetic traits that provide advantage at young age may become disadvantageous in later age. This is a kind of trade-off between increased chances to survive the period of childhood and adolescence and reproduce; and the chances to survive long after the reproductive plans have been completed (antagonistic pleiotropy). The idea of the trade-off of genetic traits at different life stages has been first expressed in 1957 in an attempt to explain the phenomenon of ageing [1189]. According to Williams, longevity and successful ageing were dependent on a set of pleiotropic genes and the different allelic forms of a pleiotropic gene might confer an advantage at one age and a disadvantage at another age. An advantage during the period when successful reproduction is likely to occur (young age) would increase the chances for passing these variants to the offspring, regardless of the fact that they may be associated with a disadvantage in later life. This results in positive selection and retention of those alleles in the gene pool. It makes perfect sense even in modern terms, as the allele pool diversity in most populations is maintained mainly by passing pre-existing alleles on to the progeny (provided, of course, that the carriers of these alleles are fit enough to reach maturity and reproduce). Williams exhibited remarkable insight, considering that in the 50-ties of the XX century the foundations of modern genetics have hardly been laid. Indeed, it is common fact of life that many individuals that have had a normal thriving childhood, have been generally healthy in their young age and produce healthy offspring, may become seriously ill or die before reaching middle adulthood, while at the same time others that were not that healthy throughout their life may reach very old age. Williams' initial hypothesis may be developed further. It is likely, for example, that the short life expectancy of pre-modern people eliminated the individuals affected with certain multifactorial diseases and conditions *before* these diseases would become symptomatic, but usually *after* the carriers have passed their genes to the offspring. This may refer to true disease alleles (such as genetic variants associated with development of hypercholesterolemia) or alleles that are confer increased risk to condition/s that may, in turn, set the stage for development of other diseases. An example of the latter is obesity. Obesity is a major health problem worldwide and much research effort is being invested in unravelling the mechanisms that contribute to it, as it increases the risk for development of many common diseases, including atherosclerosis, diabetes type 2, cardiovascular diseases and cancer. The prevalence of obesity in modern societies may reach the impressive 30–35% in adults and 15–18% in children according to data from the US Centers for Disease Control and Prevention(CDC data obesity adults; CDC obesity data youth). Of course, this is associated with the eating patterns and the decreased physical activity of modern people, but it is a common fact that obesity tends to run in families. A 'thrifty gene' hypothesis has been formulated, proposing that genetic variants conferring predisposition for storage of fat depots have been specifically selected for during human evolution [1190-1192]. Availability of food has been a major limiting factor in the development of human populations. Throughout the evolution of humankind, it was typically the individuals who had the inherent capacity to store large depots of fat when food was plentiful ('thrifty' phenotype) that could survive periods of famine. The lean metabolism has, therefore, been a kind of

luxury throughout human history, as the life of the carrier individuals was dependent on continuous availability of food. The 'thrifty' phenotype may result from mild insulin resistance, as hyperglycaemic states are associated with inhibition of lipolysis and accumulation of fat into adipocytes. When food supplies run low and the glycogen supplies are exhausted, the stored triglycerides are hydrolysed, releasing glycerol and fatty acids to be used as fuel by the glucose-starved cells. This may allow the individuals with the 'thrifty' phenotype to survive and pass their genes on, whereas less 'thrifty' metabolisers may die during famine or at least their reproductive fitness would be seriously impaired. Therefore, ability to accumulate energy for later use must have been an asset almost throughout the evolution of mankind, as plenitude of food is actually quite a recent acquisition, and is still confined largely to developed countries. The carriers of the 'thrifty' phenotype, however, would be at an advantage only as long as the average life expectancy in the population does not exceed middle age, as insulin resistance precedes the development of overt diabetes type 2 and atherosclerosis with years and decades, but once they develop, the associated complications would be likely to outweigh any selective advantages hyperglycaemia may have. Therefore, as long as living into old age was rare among humans, the 'thrifty' phenotype was advantageous to its carriers. Since the middle of the XX century, however, the life expectancy has been steadily increasing in almost all populations and especially in developed countries. Therefore, the evolutionary advantage that has been specifically selected for many centuries may eventually turn to a serious disadvantage in the modern man. In humans of today, the 'thrifty' phenotype is not viewed as advantageous or beneficial in developed countries and may be a source of health concerns.

As many genetic diseases and conditions that in the past resulted in early death or disability may now be cured or at least managed, today, genetic variants that were selected against in the past have significant chance to contribute to the genetic pool. This means that the structure of the human genetic pool is now being modified in real time and more diversity is currently being injected in human populations. The proportion of genetic variants associated with longevity and/or successful ageing may also be currently increasing, as the selection process is no longer directed solely by the capacity to survive environmental adversity.

Diseases and conditions typically associated with advanced age may sometimes occur in the young. For example, insulin resistance and cancer usually develop in middle and advanced age but may also develop at young age. This is also often misinterpreted to be a result of modern lifestyle, and, especially in popular media, to 'accumulation of environmental toxins' – whatever the latter may mean. This is a prime example of a post hoc fallacy – believing that if an event happens directly following another event, the latter is a consequence of the former. Indeed, the environmental burden with toxic agents has increased in the last decades, but it is rather naive to believe that the prevalence of human disease has increased only because of this. Rather, the diagnostic methodology has improved significantly in the recent years and the screening techniques allow for higher sensitivity and specificity in identifying individuals at risk for developing certain diseases and conditions even at preclinical stage.

7.5. Role of the genetic background in the constitution of the phenotype of successful ageing and longevity in humans

It is currently believed that about 30% of the factors contributing to longevity are heritable [1193,1194]. Several dozens of 'neutral' polymorphisms (that is, not associated with any recognisable disease or condition) have been identified in the nuclear and the mitochondrial genome in man. It has been proposed that carriership of some of these polymorphisms may subtly modify the phenotype of ageing and modulate the risk for development of some age-related diseases and conditions. For some, an effect on longevity in general has been reported. Of course, carriership of any of these polymorphisms does not have significant value in prognostication except as a risk modifier.

7.5.1. Impact of polymorphisms in the mitochondrial DNA on ageing and longevity

Cells produce, store, convert and utilise energy to live. The most commonly used type of energy to maintain the metabolism of living cells is chemical energy, produced by oxidation. About 90% of the oxygen consumption in the cell is related to oxidation of various substrates in. Since it is the mitochondria that store and handle the energy sources of the cell and implement energy conversion, it is understandable that they would bear the major oxidation impact from free radical species, generated by oxidative phosphorylation. One of the main mechanisms for activation of apoptosis is triggered by events occurring in mitochondria. Mitochondrial DNA is more vulnerable to damage than nuclear DNA, as, on the one hand, it is topologically close to the source of oxidative stress and, on the other hand, it is not packed in histones, which facilitates the occurrence of damage [1195,1196]. The negative charge of the inner mitochondrial membrane easily attracts lipophilic cations, some of which may be genotoxic – for example, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [1197,1198]. Mitochondrial DNA is inherited only from the mother's side [1199]. Usually, all mitochondrial DNA molecules in a cell are identical with regard to DNA sequence (homoplasmy), but very rarely, mitochondrial DNA molecules with different sequences (heteroplasmy) can co-exist in a single cell [reviewed in 1200]. It is believed that at some point in development the population of mitochondrial molecules in the cell dwindle down to only one molecule or, rarely, more than one (mitochondrial bottleneck), which later serves as a 'mother' molecule for synthesis of all mitochondrial molecules within the cell [1201]. The experimental data about when this 'bottleneck' occurs during development – whether it is during early divisions of the zygote; during genesis of primary oocytes in the female foetus; or during folliculogenesis in later life; is still controversial [1201,1202]. Mutations in the human mitochondrial genome are thought to occur every 6,000–12,000 years (one mutation per 300–600 generations) [1203,1204]. This is the base for calculation of the common mitochondrial DNA ancestor (see below for details). Other authors believe that the mutation rate is much faster, up to one per every 30–60 generations [1205,1206].

Role of mitochondrial polymorphisms and mitochondrial haplogroups in ageing

As the gene density in the mitochondrial DNA is very high (which is believed to constitute evidence supporting the hypothesis of the endosymbiotic origin of mitochondria), there are very few non-coding sequences in mitochondrial DNA where a nucleotide change would not have direct impact on the phenotype. Most alterations of the mitochondrial DNA sequence

are associated with severe pathological phenotypes, such as Leber hereditary optic neuropathy (LHON); Leigh syndrome; myopathy with ragged red fibers (MERRF); MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes); maternally inherited DAD (diabetes and deafness) syndrome, and others. However, heritable polymorphic variants in mitochondrial DNA do exist. The sites where alterations in mitochondrial DNA may occur without immediate harmful effects (predominantly around the control region in mitochondrial DNA, specifically the D-loop) are termed hypervariable regions (HVRs). Since mitochondrial DNA does not engage in recombination during meiosis, virtually all mutation occurring in it may become fixed. In case the mutation is not deleterious, it may be transmitted to the progeny of the cell during mitosis as well as down the generations. Some mitochondrial polymorphisms may modulate the amount of oxidative stress generated in the cell and/or the capacity of the cellular repair machinery to handle it safely. A set of polymorphisms within the same DNA molecule is commonly called a haplogroup. Carriership of different haplogroups of mitochondrial DNA may be associated with differential rate of physiological ageing and differential risk rates for development of certain age-related diseases and conditions. Specific mitochondrial haplogroups may also play a role in longevity (see below).

The common origin and the subsequent divergence of different mitochondrial haplogroups may be traced with considerable accuracy for hundreds of generations back in human history. Mitochondrial lineage, however, may only be traced along the maternal line, as mitochondrial DNA is only transmitted by the mother to her children. According to modern theories, the origin of all people currently living on Earth may be traced back to a single female ancestor, commonly called 'Mitochondrial Eve' (mtEve)' [1207]. "Mitochondrial Eve" is the female counterpart of the so-called "Y-chromosomal Adam", the common ancestor of all modern males. The Y-chromosomal Adam is believed to have been the source of the Y-chromosome from which diverged virtually all Y-chromosomal types existing today. Of course, this rather romantic idea of Adam and Eve does not imply that at some point in history humankind consisted of one man and one woman only. Even the earliest human populations comprised at least several hundred to several thousand members at any given time, and were made of roughly half-and-half men and women. Potentially, any of these women could have become the mitochondrial Eve. With time, however, some women produced offspring that consisted only of boys and there was no daughter to transmit the mitochondrial DNA to the next generation; and some had daughter/s, but they died childless. Thus, in the majority of the women who potentially could have become mitochondrial Eves, the maternal line had been broken at some point. Similarly, the Y-chromosomal Adam is not one man who became father of the human race, but a human male in whose descending line there always was one fertile son per generation to transmit the Y-chromosome to the next generation. The number of possible mitochondrial Eves and Y-chromosomal Adams eventually dwindled down with time to one per gender that provided the ancestral mitochondrial genome, variants of which we all carry today and the ancestral Y-chromosome that with small variations was transmitted to all currently living men. The mitochondrial Eve and the Y-chromosomal Adam had never actually met each other, as they lived about 50–100,000 years apart. It is believed that the 'mitochondrial Eve'

had lived in Eastern Africa about 200,000 years ago [1208,1209]; and the Y-chromosomal Adam probably lived in Africa too, but much later, about 60–150,000 years ago [1210,1211]. Different mitochondrial haplogroups may be graphically presented as branches of the phylogenetic tree originating from the mitochondrial Eve (Fig. 23). Each of the mitochondrial haplogroups had arisen at different times in human history, typically separated by tens of thousands of years. It is believed that the 'mitochondrial Eve' had seven 'daughters' (meaning – it initially branched out into seven subgroups), one of which, called 'Lara' produced the first of the ancient haplogroups, L (from 'Lara') [1212]. 'Lara' is believed to have lived about 100,000 years ago in Africa and had herself produced 8 branches – from L0 to L7. Haplogroup L3 produced by divergence all currently existing mitochondrial haplogroups (Fig. 23).

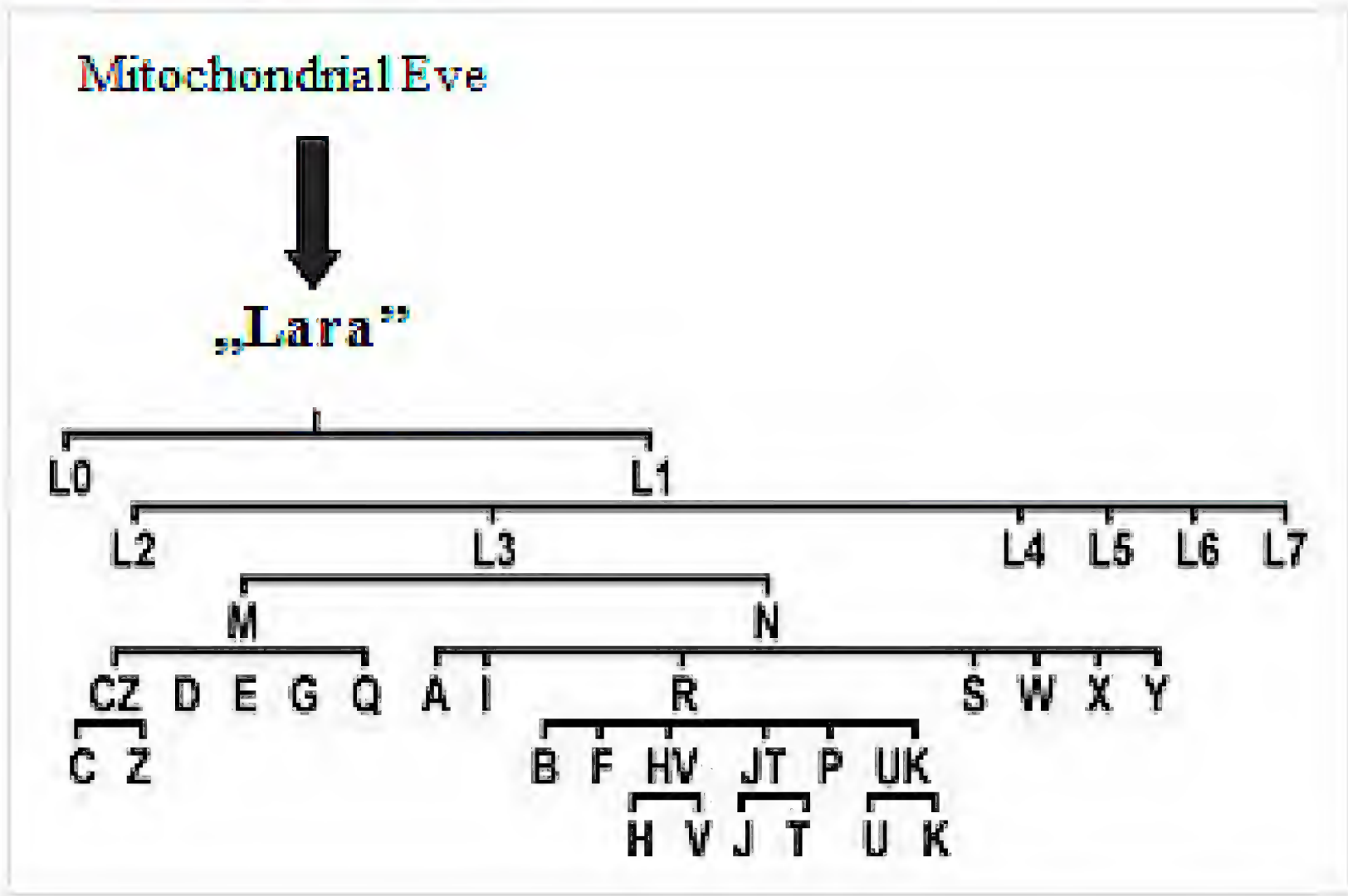

Figure 23. Divergence of different mitochondrial haplogroups from the mitochondrial Eve. The branches are usually denoted with Latin letters (A–Z), but the degree of relatedness between them does is not reflected by alphabetical order.

In present day, there are 8 major mitochondrial haplogroups which may be seen in Europe and several others that are more common in other continents. Basic data about major mitochondrial haplogroups in Europe may be seen in Table 6.

Table .6 Trivial names and likely times and places of origin of modern mitochondrial haplogroups commonly seen in European populations [1212]. Haplogroups for which no percentages are given are very rare in Europe.

Haplo-group	Trivial name	Possible time of origin [years ago]	Possible place of origin	Prevalence in European populations [%]
N		≤ 75,000	South Asia	
R		≤ 70,000	South Asia	

U	Ursula	≤ 60,000	North-eastern Africa or Southwest Asia	about 10
JT		≤ 50,000	Middle East	
J	Jasmin	≤ 45,000	Near East or Caucasia	about 15
HV		≤ 40,000	Near East	
H	Helena	≤ 35,000	Western Asia	about 50
X	Xenia	≤ 25,000	North-eastern Europe	5 - 6
I	Iris	≤ 25,000	Caucasia North-eastern Europe	
W		≤25,000	North-eastern Europe North-western Asia	
K	Katrine	≤ 15,000	Near East	5 - 6
V	Velda	≤ 15,000	Iberia (currently, on the territory of Spain)	5 - 6
T	Tara	≤ 15,000	Mesopotamia	about 10

The oldest haplogroup of these that may be seen today is haplogroup N, branched out from L3 about 75,000 years ago. The newest of the modern haplogroups is T (arisen from JT about 15,000 years ago) [1213]. Similarly to 'Lara', most mitochondrial haplogroups of today have trivial women's names as well.

A simple schematic of the divergence of the mitochondrial haplogroups as mankind spread out from Africa to Asia and Europe (60–125,000 years ago), then to the Americas and Australia (about 40,000 years ago) is shown on Fig. 24.

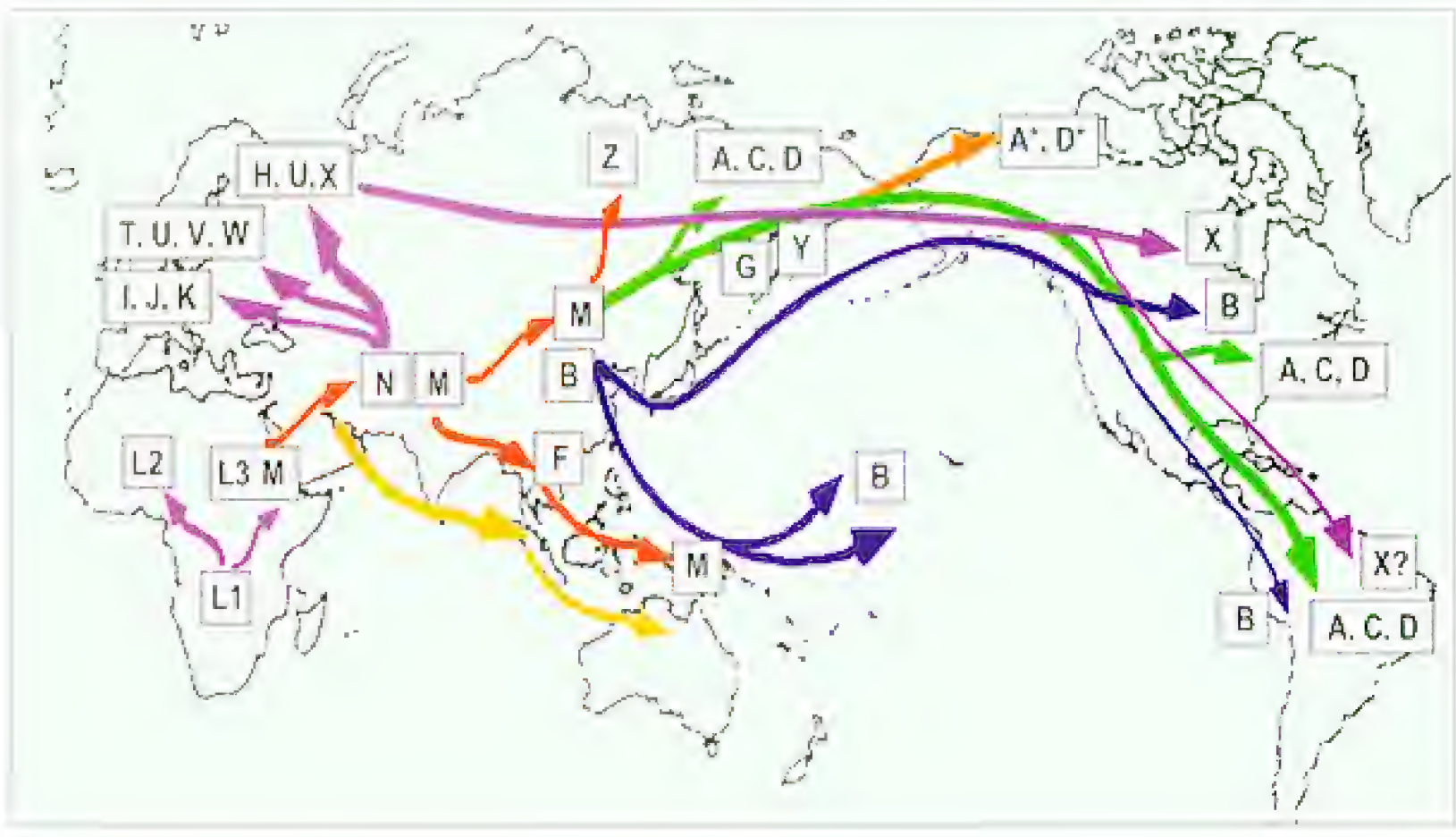

Figure 24. Divergence of modern mitochondrial haplogroups.

The calculations about how long ago have our common mtDNA ancestor lived are based on the premise that a mutation in mitochondrial DNA occurs roughly once in 300–600 generations. There are other studies that report mutation rates about 1:30–60 generations, dating the 'mitochondrial Eve' to a mere 6,000–6,500 years ago [1205,1206].

Usually the most commonly seen haplogroup in people of Indo-European (Caucasian) origin is H (Helena) – about 50% in all European haplogroups, followed by J (Jasmin) – about 15%, U (Ursula) and T (Tara) – each about 10% of the population. I (Iris) is the rarest haplogroup

in Europe– under 2%. Every haplogroup has several minor branches (subclades). Different subclades may have different prevalence in different countries. On the British Isles, H1 accounts for over 10% of all haplogroups, except in Ireland where most common is the subclade U5 (10%), followed by H3 (6%) [1214]. H3 usually has the highest prevalence in Central Europe, about 5–6%. In Southern and Eastern Europe, including Bulgaria, the most common mitochondrial subclade is H2a [1215]. In the aboriginal population of territories which had been geographically separated from Europe long ago (e.g. the Americas) the most common haplogroups are usually A, B, C and D (generally not seen in Europe) and the recently defined haplogroup X (Xenia) that may be seen in European populations, but at low prevalence.

The importance of mitochondrial haplogroups in human ageing was first noticed in research on centenarians. It was repeatedly observed that some mitochondrial haplogroups were more commonly seen in people over 90 and the differences in the prevalence could be explained by pure chance. For example, the increased prevalence of haplogroup J among individuals living beyond 85 has been demonstrated in studies in several different populations (although not in all populations) [1216-1218]. It was considered rather curious, therefore, that genetic mutations in a variety of mitochondrial genes resulting in Leber hereditary optic neuropathy were found more often in carriers of mitochondrial haplogroup J [1219]. Also, carriership of J haplogroup in patients with multiple sclerosis increased the risk for development of optic neuritis [1220]. Accumulation of oxidative stress in mitochondria may actually account for both phenomena. It was experimentally proven that mitochondria carrying DNA of haplogroup J have the lowest oxygen consumption of all haplogroups [1221,1222]. On the other hand, lower oxygen consumption is associated with lower levels of reactive oxygen species (ROS) generated by oxidative phosphorylation. Therefore, the levels of oxidative stress in mitochondria of haplogroup J are consistently lower. This may explain at least partially the higher life expectancy of carriers of mitochondrial haplogroup J. On the other hand, it is believed that higher oxygen consumption characteristic of haplogroups other than J may somehow compensate for some of the molecular defects associated with LHON and the risk for multiple sclerosis. Thus, these two diseases are more likely to become manifest in carriers of haplogroup J, as the phenotype is not ameliorated by higher oxygen consumption [1220,1223].

Haplogroup X (Xenia) is another haplogroup that is often seen in healthy octogenarians and nonagenarians. It has been found to be associated with the phenotype of 'successful ageing' – that is, ageing in which the capacity for normal physical activity, the memory and mental clarity are completely preserved into very old age (for details, see below) [1224].

Haplogroup H, which is the most common mitochondrial haplogroup in Europe, is associated with the highest mitochondrial oxygen consumption of all haplogroups [1221]. An association has been identified between carriership of haplogroup H and diabetes type 2, but it does not affect the risk of development of the disease, but, rather, the risk of certain complications, specifically retinopathy and neuropathy [1225].

The risk for vascular incidents may be modulated by carriership of different mitochondrial haplogroups. For example, for carriers of haplogroup A the risk for vascular incidents in middle and advanced age may be higher than the population risk, while in carriers of the

subclade N9a of haplogroup N the risk may be lower [1226]. At least in some populations, subclade N9a has been found to be protective against other age-associated diseases and conditions as well. For example, diabetes type 2 and metabolic syndrome seem to be less common in Asian carriers of N9a [1226,1227].

Role of accumulation of damage in mitochondrial DNA in ageing and age-related disease

All proteins involved in repair of mitochondrial DNA are coded by nuclear proteins and may be identical in structure to their nuclear counterparts or slightly different (for example, mitochondria-specific isoforms resulting from alternative splicing of the mRNA coded by the nuclear gene). The profile of DNA repair in mitochondria, however, may be very different from the profile of repair of nuclear DNA. For example, repair by excision of nucleotides is virtually absent in mitochondria [20,1228]. As we already know, NER is the most versatile repair mechanism, capable of repairing virtually any kind of damage, so its absence in mitochondria may seem rather odd. Repair by base excision is, however, maintained at normal levels in mitochondria [1229,1230]. Oxidation of bases is the most common type of DNA damage in mitochondria, and the damage is readily repaired by BER. It makes sense, therefore, to assume that NER may become redundant in mitochondria, as there is a designated mechanism for repair of the most common type of lesions and mitochondrial turnover is rather rapid in normal cells anyway. Mitochondrial BER may be additionally stimulated under conditions of increased oxidative stress. For example, the human homologue of the bacterial N-glycosylase Fpg –*hOGG1*, removing oxidised bases from mitochondrial DNA as well as the nuclear DNA, was shown to be up-regulated in response to ischaemic stress and drug-related oxidative damage in neurons [1231,1232].

It is currently believed that chronic oxidative stress in mitochondrial DNA plays a major role in the pathogenesis of some age-related diseases, such as retinal degeneration [1233,1234]. It is believed that the loss of subcutaneous fat, which is common in advanced age (and is one of the typical features of progeroid syndromes), is due to mass apoptosis of adipocytes, triggered by oxidative damage to their DNA [1235]. Oxidative damage has been found to play a role in ageing not only on DNA level but also by oxidation of various signalling and effector proteins. For example, the mass death of selected cell populations in specific regions of the brain observed in idiopathic Parkinson's disease has been found to be triggered by oxidative stress [1236]. It is believed that this is at least partly related to the excitotoxic action of the oxidised dopamine derivative 6-hydroxydopamine, which triggers the endogenous pathway of activation of apoptosis by stimulating the release of cytochrome c from the mitochondria [1237].

Carriership of mitochondrial haplogroup K has been found to be associated with increased levels of oxidative stress, sustained levels of mitochondrial DNA damage and higher risk for breast cancer [1238].

7.5.2. Impact of polymorphisms in the nuclear DNA on ageing and longevity

Honour thy father and mother;
... That it may be well with thee,
and thou mayest live long on the earth.
Ephesians, 6:2–3

Current experimental data show that genetic factors play a role in longevity, although the definitions are somewhat fuzzy and the weight of the individual factors cannot be estimated precisely. It is difficult to formulate, for example, exactly long is a very long life. In countries where the average life expectancy is 75 years, 85 years are a very long life, but in countries like Japan, where the life expectancy is over 82 anyway, 85 years is only average. The average life expectancy in a population gives a fairly reliable estimate about the population, but does not provide grounds for individual prognostication. Average life expectancy does not allow differentiation of specific sub-populations where the life expectancy may be different from the average (e.g. families with increased rate of cancer due to a founder mutation or long-lived families in which life expectancy is different from the population average but is similar between members of the family). It is known that long-lived people usually come from families in which one or more members lived to very advanced age, but individual prognostication of life expectancy is unreliable at present, except in very broad limits. This is mainly because some of the most commonly used criteria are unspecific (e.g. presence of harmful habits – smoking, drinking, diet laden with carbohydrates and fats, etc., that would be disadvantageous to anyone); too broadly defined (e.g. 'stress'); or based on unreliable information (e.g. 'Did you parents have had or do they presently have any of the following medical conditions', to which many people would reply 'I don't know' or 'I don't know what these terms mean'). At present, the only factor in the constitution of life expectancy that has something to do with individual status is the age at which one's parents died. Specifically, it was found that people whose relatives from the direct line (parents, grandparents) lived at least 90 years were likely to live longer than the average life expectancy of the population [1194]. Of course, it refers only to deaths 'of natural causes' and not deaths related to motor vehicle accidents and natural disasters. Certainly, some of the very long-lived individuals in all populations come from families that had average life expectancy, but the chance for living longer was found to be greater for people with at least one close relative that died at age >90 than people without long-lived relatives from the direct line. The age to which parents lived is a very simple empirical criterion, but it seems to predict more reliably the individual life expectancy than an inventory describing one's habits and stress levels. What is curious, however, is that the term 'parents' in this case may refer to biological as well as foster parents. Individuals that have been adopted by long-lived parents may have life expectancy above population average [extensively reviewed in 1239]. Clearly, the familial pattern of life expectancy may be modified by environmental components as well (e.g. lifestyle habits, family traditions, living in areas relatively free from industrially pollution areas, etc.). The age at which relatives other from parents died may also play a role in constitution of individual life expectancy. Twin studies show that the ages at death of monozygotic twins are on the average 3 years apart, while in dizygotic twins the average variance in ages at death is about 6 years, that is, the same variance that is observed between siblings. Again, this is valid only for death 'of natural causes' [1240,1241].

Carrying a genetic variant associated with disease may reflect on one's life expectancy – usually, by decreasing it. To the present moment, several loci have been identified that are associated with life expectancy without predisposing to disease, or, at least, not directly. It

has been reported that in humans >85 years of age, individuals homozygous for the proline variant of p53 at codon 72 exhibited a significantly (>40%) enhanced survival compared to Pro/Arg heterozygotes and individuals with Arg/Arg genotype [1242]. At the same time, the Pro/Pro individuals in the cited study had a >2-fold increase in the incidence of cancer. A couple of years later, it has been shown that in some populations the healthy homozygotes for the Pro allele exhibited a consistent increase in median lifespan, measurable in years, compared to homozygotes for the Arg allele [1243]. It has been proposed that, since the risk of cancer is actually higher in the Pro/Pro group, the increased life expectancy may actually be due to increased survival after a diagnosis of cancer or other age-related disease. None of the two alternative forms of TP53 codon 72 polymorphism is associated with any type of disease. The fact remains, however, that the carriers of the Pro variant tend to live longer than the carriers of the Arg variant of *TP53* [1244,1245].

Two genetic loci associated with longevity have been identified so far – *LNGV1* (4q25) and *LNGV2* (*FOXO3A*, 6q21) [1246,1247]. Several distinct allelic variants of the *LNGV1* gene were found to often segregate in families with many long-lived members as well as in individuals who 'age gracefully' (or 'successfully', see below) [1248,1249]. No genes associated with any type of disease or condition had been found in the *LNGV1* locus and its potential connection to longevity was identified by association studies only [1246]. Indeed, in a neighbouring locus to *LNGV1* lies the *CISD2* gene (4q24), mutations in which produce a distinct disease phenotype in humans – namely, Wolfram syndrome type II [1250,1251]. *Cisd2* knockout mice are characterised by a phenotype of accelerated ageing, including premature loss of subcutaneous fat, wrinkled skin, greying fur and cataracts [1252]. Mouse cells with knocked out *Cisd2* gene copies exhibit rapid mitochondrial turnover. No polymorphisms in the *CISD2* gene associated with human longevity have been identified so far. The *FOXO3A* (*LNGV2*)gene codes for a G2-M checkpoint protein activating the transcription of repair proteins via GADD45. The latter is a downstream target of BRCA1/2 protein complex, inhibiting the entry into the S phase [882]. FOXO3A also increases the levels of mRNA and protein of Mn^{2+}-dependent superoxide dismutase in non-dividing cells, thus providing protection oxidative stress [1253].

In 2010, in the promoter of the *ATM* gene was identified a single nucleotide polymorphism that was commonly found in Asian nonagenarians and centenarians [1254]. It is believed that genotype by this polymorphism is related to the capacity for response to DNA damage. The effect on longevity was observed in heterozygotes only.

Three novel loci associated with longevity were identified by linkage analysis on chromosomes 3, 9 and 12 [1255,1256].

Telomere length is a marker of the proliferative capacity of eukaryotic cells. Twin and family studies show so far that the mean telomere length in man (measured in leukocytes from peripheral blood) is actually heritable –parents with longer telomeres usually had offspring with longer telomeres [1257,1258]. A rather curious finding is that the linkage between advancement of age and the length of telomeres in certain types of cells and tissues may be actually positive. Specifically, it has been demonstrated that the length of telomeres in the offspring could be directly related to the age of the father at the time of birth of his children – the older the father was, the greater the average telomere length in peripheral leukocytes

in his children [1259-1261]. It was demonstrated that despite the fact that the telomere length measured in peripheral leukocytes decreased with increasing age, the value was not directly related to the mortality rate, especially after the age of 70 [1262,1263].

7.6. Ageing and 'death of old age' as an adaptive mechanism

A person often meets his destiny on the road he took to avoid it.

Jean de La Fontaine (1621–1695)

7.6.1. Our life (and death) are preprogrammed

Life cycle of higher animals, including humans, usually follows a predefined timeline, starting with a thriving childhood and adolescence, followed by a relatively steady period during which reproduction is ensured and care of the offspring is taken, and, eventually, around the middle age and old age, the population experiences an increase in morbidity and mortality. It has been theorised that as the development and growth of an organism may be programmed, so are ageing and death [1147]. According to this theory, ageing is not just the unavoidable result of many random 'hits', (albeit they do play their role), but a part of the normal life cycle of cells and organisms.

In humans, there are some age-related changes which are apparently preprogrammed and are only partly dependent on external factors. For example, the menopause in women and the associated hormonal and functional changes are not simply results of 'getting old', but follow a predefined course. The attrition rate of ovarian follicles begins to rise approximately 10 years before the cessation of menses and this may only partially be modulated with medication. The onset of menopause may be delayed by hormonal therapy and the associated unpleasant experiences may be ameliorated, but the process cannot be avoided or brought to a standstill. In this case, the 'age of parent matters' rule is gain valid; as menopause in most women starts around the age it had started in their mothers.

Curiously enough, in some higher mammals such as dogs and cats, the menopause seems not to be programmed into the life cycle of the female. Studies on domesticated cats and dogs which live much longer than their 'wild' counterparts show that they have no equivalent of menopause, that is, the oestrus will repeat with regularity (more or less) until the death of the female animal, even at age which is very advanced for the average life expectancy for the species and even though the animal is unable to conceive and carry a potential pregnancy safely to term. It has been discussed that this may be related to the life expectancy in the wild, which may be several times lower than in domestic animals. 'Wild' (or, rather, free-living) dogs and cats live on the average 7–8 years, whereas domestic cats and dogs often live up to 12–15, sometimes even 18–20 years. Apparently, Nature never meant wild animals to live beyond the age in which offspring could be safely conceived, carried to term and brought up to (relative independence). Humans, however, must care for their children for over a decade after they have been born. It might have been preprogrammed that the adults would live beyond the age in which they can create new babies, so as to be able to take full care for their offspring until they have achieved the maturity needed to become parents and caretakers themselves.

7.6.2. Why may ageing be actually good for us

Accumulation of DNA damage and mutations in DNA is now known to be a major mechanism in ageing. At first glance, the optimal mode of action in order to delay or arrest

cellular ageing is pretty straightforward – making sure that the cells are maximally protected from the impact of DNA damaging agents and ensuring that the DNA repair machinery promptly recognises and corrects each every error in time. Turns out, however, that is unlikely ever to work. A significant amount of the DNA damage that the cell needs to manage daily is generated by normal physiological processes and cannot be minimised or even decreased safely without serious consequences for the cell's normal functioning. Also, consistent, maximum-precision repair of all errors that occur in the cellular DNA would mean that the genetic information of the progeny of a cell would be exactly identical to the information in the mother cell. In the short term (throughout the lifetime of the cell), this may be not harmful per se. After all, eukaryotic cells have more than one mechanism for assessing the position of the cell in the timeline of individual existence (e.g. the telomere attrition mechanism). A cell that had reached a critical telomere length would normally enter replicative senescence and die even if its DNA is in pristine condition. In terms of the life of the multicellular organism, accurate repair of all DNA errors may still be a good thing, as it may increases the chances for health throughout the life of the organism and, possibly, for longevity. Nature, however, does not operate on individual scale. If every new living thing (be it a cell or an organism) is exactly the same as the living thing/s before them, they would not be capable to adapt to potential changes in environment. In other words, the cell or the organism may benefit from extremely accurate DNA repair as long as they exist for their own sake only. When they produce offspring, however, supplying them with the very same genes as the parent organism may not be quite as beneficial, as the environment is likely to change as time goes, and what worked for the parents may not work for the offspring in the long term. Thus, either we change and die, or stay as we were and die all the same.

It has been proposed that ageing could be an adaptive mechanism, specifically developed in the course of evolution. By definition, an adaptive mechanism must confer some selective advantage, or else it will not survive evolution pressure. At first glance, ageing has not much to offer – a gradual post-reproduction decline, ultimately ending in death. Looking deeper, however, may reveal that ageing offers not one, but two significant advantages, at two hierarchical levels – protection from cancer (for the individual) and protection of the genetic diversity (at population and species level). The advantages of the first level are fairly obvious. 'Death of old age' is usually multisystem failure, caused (directly or indirectly) by gradual depletion of the regenerative capacity of the tissues and organs. As age advances, the risk that one or more organs or systems would fail increases. The risk for cancer, however, also increases with age. Thus, ageing may serve to restrict the capacity for division of cells which have already been exposed to genotoxic agents (exogenous or endogenous) for long enough time. Ageing cells are forced to divide less frequently and more slowly, thus decreasing the risk for accumulation of too many mutations to unleash cancer growth. The more complex an organism is, the greater the risk for development of cancer with time, as complex organisms contain many different types of cells, in any of which the mechanisms for restriction of uncontrolled cell growth may be bypassed or abrogated in different ways. Immortality (or living a very long life) would, therefore, be unavoidably associated with development of cancer.

The second line of advantage that ageing offers – namely, maintenance of genetic diversity – is directly related to the reproductive decline associated with ageing. In species in which there is no preprogrammed menopause, the young are usually capable to take basic care of themselves (e.g. finding food and shelter) weeks, months, an year or, sometimes, a couple of years after birth. Therefore, even if the parental generation dies early or becomes unavailable for any reason, the offspring is likely to reach sexual maturity and reproduce, so as to keep the population going. In species with preprogrammed menopause (specifically, humans, but also chimpanzees [1264]) the capacity to bear young is usually lost long before the natural lifespan is finally over, so that no adult organism is allowed to contribute too much to genetic pool of the population. Of course, every individual that has reached sexual maturity is given a chance to reproduce and transmit their genes down the generations, but only within a limited timeframe, which is usually in youth to early middle age. Thus, the mechanism of ageing permits procreation only to those that have lived long enough to be capable to create and bring up viable progeny, but not too long so as to have too mutations accumulated in the somatic cells and the germline. The risk for passing mutations to the progeny would presumably be too great, potentially causing disease or death before the young have acquired the capacity for independent life.

As the normal functioning of p53 is very important for the life (and the death) of the cell, one could conclude that keeping the p53-regulated pathways primed, alert and functional must be the safest way to ensure that the cell (and, respectively, the organism) are kept cancer-free. In this train of thought one could presume that as such cells and organisms would more rarely succumb to cancer, they would live longer. Research of the association between p53 and longevity, however, suggests that the lifespan of cells and organisms with an 'over-zealous' p53 would not probably be a very long one. Mice carrying mutations in the *TP53* gene associated with constitutive activation of p53 (deletion of the first six exons of the TP53 gene) showed enhanced resistance to development of spontaneous tumours compared with wild type mice. These mutant mice, however, displayed an early onset of ageing-associated conditions, such as osteoporosis, generalised organ atrophy and diminished stress tolerance, and had a noticeably shorter lifespan compared to their wild type littermates [1265]. In mice in which the gene coding for the major negative regulator of p53 – MDM2 has been deleted in the cells of the epidermis, the characteristic ageing phenotype was also promptly observed, including epidermal thinning, impaired wound healing, and progressive fur loss [1266]. Reduced p53 activity in aged animal models was associated with higher numbers of proliferating haematopoietic stem and progenitor cells compared with wild type controls of same age [1267]. It seems that prompt functioning of anti-inflammatory and tumour-suppressing mechanisms such as those related to p53 are indispensable in young age, ensuring that the organism is cancer-free and fit as long as it is capable of producing and rearing offspring. In older age, the likelihood for disease-free survival is somewhat at odds with longevity. On the one hand, multiple cycles of cell division required to sustain the tissues throughout a very long life increases the risk for introduction of heritable mutations, and, respectively, the risk for cancer. On the other hand, prompt disposal of cells that may potentially give rise to a malignancy would, with time, deplete the stem cell stores, thereby reducing the potential for cell and tissue renewal

of the ageing organism. The latter is associated with development of age-related degenerative disease. p53 keeps the genome integrity in check by disposing of damaged cells, but the same mechanism is responsible for cutting down the disease-free survival of the organism beyond young and middle age by triggering the ageing process [reviewed in 1268].

Ageing makes sure that individuals (be it cells or people) have a roughly even chance to contribute to the genetic pool but that once the reproductive age is over, they are allowed to live only as long as they do not constitute a potential threat to the genetic diversity of the population. All very rare instances of 'immortal' eukaryotic organisms currently known to science are historically very old and with very simple organisation (the already mentioned Cnidaria). It is notable that the equivalent of cancer has not been described in Cnidaria. Presumably, their phylogenetic branch was the last in Earth evolution in which it was possible to maintain the prokaryotic property of living for a very long time (practically, forever), producing many identical generations, and not suffer the consequences.

7.6.3. The tremendous cost of living forever

I may be destructible, but I am not simply mortal. My organs, tissues,
even the molecules that make them up function at equilibrium
so perfect that the forces acting within me could never, ever
shift this equilibrium apart. I am not longer an individual, but a
species of one, not a person, but a category, for I am – complete.
S. Snegov, The Experiment of Professor Branting (1977).

At least in theory, natural wear and tear of tissues and organs could be compensated by maintaining the production of new cells to replace the old ones. In complex multicellular organisms, this may potentially be achieved by stimulation of the adult stem cells to produce specialised cells. What could be gained – purely hypothetically – if constant renewal of cells and tissues was actually possible? Let us imagine a population of DNA- and protein-based life forms with inherent capacity to renew aged or otherwise damaged cells. These beings are very close to the model commonly called a Darwinian or Darwin's demon – a hypothetical organism capable of maximising all aspects of biological fitness. Darwin's demon is actually a derivative of Maxwell's demon, a hypothetical automaton capable of producing order locally from chaos [1269,1270]. In order to avoid occurrence of harmful mutations, these beings must have extremely high-fidelity system for DNA synthesis during replication and repair, ensuring that DNA of every cell would pass intact to the cell's progeny during division. This would have two immediate consequences. The first consequence is that the population members would have the potential for living for an indefinitely long time, practically forever – unless a serious injury or some type of severe physical deprivation (air, water, food, etc.) renders them unfit to live. The second consequence would be that, if our hypothetical beings are capable of sexual reproduction, they would never experience age-related hormonal decline/menopause, as the gamete-producing cells, too, would maintain their DNA in pristine condition. Thus, our hypothetical living beings would be capable of reproduction from the moment they reach sexual maturity for a very long time onwards, practically forever. Let us then assume that our immortal population lives in a relatively stable environment (no drastic changes in the

climate or in the composition of the air they breathe, etc., or if they occur, the intervals between occurrences are measured in very long periods – thousands of years, or even geological eras). In a relatively short time, the population would to crowd the space they live in, and they would eventually die out of lack of resources as no member would die of old age, allowing younger members to live. This would effectively put the population to an end very soon. In order to visualise the long-term consequences, however, let us assume that all resources are abundant and easily accessible, eliminating starvation and/or draught as potential reasons for population extinction. As death would be uncommon event in the population, the physiological protective mechanism for sensing and acting to prevent danger is likely be underdeveloped. Thus, some members of this population would die because of physical injury over time. This means that, in time, the majority of the population would consist of descendants of some of the members of the initial population and not others (e.g. those that have died because of accidents and injuries). This would decrease genetic diversity, and the longer time passes, the less heterogeneous the population would become. Let us remember that our hypothetical beings live for a very long time, so they would experience some changes in the environment. As the immortal beings replicate and repair their DNA with 100% fidelity, they have no mechanism to adapt to climate and geological changes. This would be, actually, the greatest problem they ever face, as features (that is, tissues and organs) that are no longer used cannot be 'removed' and features that may come useful cannot emerge. For example, breathing by gills may be helpful when oxygen comes from water but not from air, therefore, if the water basins recede or dry out, there would be no mechanism to adapt to living on dry land. As our Darwinian demons can die because of physical damage, the first drastic change in the climate may result in their extinction, as the extreme accuracy of replication and repair of DNA would not allow for testing and trying other DNA variants that may be beneficial in a changing environment. Thus, the population that, by definition, had all the potential to live forever would eventually die because of genetic stagnation. Apparently, 100% fidelity of DNA synthesis in replication and repair is not a viable option if the organism lives forever.

Let us consider then the alternative option, in which our hypothetical beings are inherently capable of introducing much diversity as possible into their genetic pool via mutability. They would still be capable of renewal of all damaged cells, but may also adapt to virtually all environments. Mutagenesis is a random process, however, so there is no guarantee that all mutations that occur would be adaptive and beneficial for the individual. Thus, the more fortunate individuals that by pure chance had become carriers of mostly beneficial or neutral genetic mutations would survive, and others who were less lucky may die, as the mutations may cause very severe disability and/or organ failure. Since the individuals that survive may live and produce offspring practically forever, after long enough time the population would consist of a limited variety of 'lucky' genotypes. The genetic diversity would decline with every successive generation, as there would be the added risk that some of the progeny in every generation may acquire de novo mutations that may be potentially lethal. Some of the accumulated mutations might have been beneficial in the past, but may become useless or even harmful later, therefore, the surviving members of the population would need to mutate continuously in order to adapt to changes in the environment. As a

result, the individual members may eventually become very different from each other with regard to genotype as well as phenotype. As sexual reproduction requires high degree of similarity between the mating individuals, it may, sooner or later, become impossible, which would intensify inter-individual divergence. Eventually, a very limited number of individuals with enormous mutation burden would survive. Each of them would eventually take an independent course of evolution, as exchange of gene variants via sexual reproduction would be impossible. Every individual would exist as an independent entity, a species of one. Development of society would, therefore, also be impossible, as individual members would have nothing in common and could not share experiences. If conscience develops, it would be purely solipsistic, as one individual would have no way of knowing that there were other individuals that could feel and think.

Immortal beings made of DNA and protein actually live on Earth in this very moment – namely, members some of the subdivisions of the phylum Cnidaria. These are very simple animals that had branched out of the common evolutionary tree about 550–600 million years ago. They are capable of both asexual and sexual reproduction. The sessile form (polyp) generally reproduces asexually, producing many juvenile forms that later develop into free-living (swimming) forms that may eventually become sexually mature, while the initial polyp may continue to grow and produce multiple identical copies by budding. Sexual reproduction is often employed in response to changes in the environment. Cnidarians can regenerate when injured, with the regeneration capacity varying among the different stages in the life cycle (the sessile form is capable of regenerating body parts and whole new organisms even from small aggregates of cells, while the swimming form (medusa) has more limited regeneration capacity). Some Cnidarians (some of the members of class Hydrozoa) are known to be capable of living for a very long time, practically forever. The mechanisms to achieve immortality may be different in different Hydrozoans, but capacity of cell renewal is essential in both cases [1271,1272]. The genus Hydra is characterised by very high regeneration capacity, allowing growth of new adult organisms even from single cells (morphallaxis). For the jellyfish Turritopsis dohrnii (formerly T. nitricula) the process resembles rejuvenation, as adult sexually mature individuals are capable of reverting back to the immature stage (polyp) by a process similar to transdifferentiation [1273,1274]. The biology of Hydra spp. is closer to our hypothetical example. The somatic cells of the asexually reproducing polyp of the hermaphroditic hydra are steadily dividing, with old cells sloughing off and being replaced about every 20 days [1275]. Hydra's somatic cells can die but they are constantly replaced in order for the multicellular organism to survive. Hydras may go on with asexual reproduction for a very long time, especially when food is plentiful. They usually revert to sexual reproduction when food becomes sparse or whenever other unfavourable changes in environment occur. The fertilised eggs are usually capable of surviving adverse environmental conditions, and the sexually differentiated adults may then experience a decline in the capacity for motion and capturing food, similar to ageing. This phase of the life cycle is characterised by a sharp rise in the mortality rate of the population [1276,1277].

Apparently, resistance to change works only in relatively stable environments. No environment on Earth remains the same forever; therefore, some genetic flexibility must

exist to ensure that life would continue to exist. Apart from spontaneous mutagenesis, the increase of genetic variability in the population is usually implemented via sexual reproduction between unrelated individuals. This comes at the price of the parent individuals eventually dying, but they leave behind a progeny with increased genetic diversity, in the hope that they would survive to become parents in their own time.

The ability to constantly produce substitutes to aged or dead cells is also not a universal solution for attaining eternal life, apart for primitive organisms. Cnidarians are very simple multicellular organisms that had given up evolution more than a half a billion years ago. It is likely that this was the period of time when the evolutionary decision to choose a general direction of development of life on Earth was made so that the potential for survival of the generations to come prevailed over the choice of a very long life for the individual. Thus, the hope for living forever was sacrificed in order to make sure that life goes on forever.

How does life turn out, however, for living beings which are, in evolutionary terms, much more complex than the single-celled organisms and Cnidarians? It had been provided that they would have a finite lifespan, during which they would have the chance to reproduce and, in higher animals and in man, to take care of their offspring so as to ensure that their descendants would survive and adapt to the environment well enough to create and raise their own young. Meanwhile, the organism would age, and, eventually, would die. The majority of the individuals in the population, however, would have had a reasonably long 'time window' in which they may contribute to the genetic diversity of the population and the species. Ageing, therefore, may be viewed as a mechanism to ensure that the 'old' constituents of the genetic pool would be removed from the population, so as the newer genetic variants would have an equal chance to be tested in the real world.

Molecular evolution is, however, still working, even at present, although it is a very slow, 'hit and miss' process. Every new generation is at risk for introduction of potentially harmful mutations. Replicative senescence (which is, for all intents and purposes, the quintessence of ageing) and programmed cell death would ensure that cells that have lived long enough and have, therefore, accumulated mutations above a certain threshold would die, so as to avoid the risk of passing altered DNA to the cell's progeny. In the rare cases when mutated cells do not die, but continue to divide, cancer occurs. Shocking that it may seem at first, ageing and cancer are currently viewed as key evolution mechanisms, large-scale equivalents of DNA repair and apoptosis that protect the life on Earth from being extinguished because of an untimely evolutionary twist.

7.7. Not getting older, getting better: the concept of successful ageing

Let Time that makes you homely, make you sage,
The sphere of wisdom is the sphere of age.
Thomas Parnell, An Elegy to an Old Beauty (circa 1710), l. 35.

All developed human populations have experienced in the last several decades an increase of the proportion of the individuals with completed reproductive plans (middle age and beyond). The popular press is currently teeming with articles sending out the general message 'the world is ageing – this means you – start worrying now' and provide a legion of tips for staying younger, some of which are frankly useless and some downright alarming (for more detailed discussion, see below). If we stop and think about it, the fact that the

population is ageing means also that our parents and grandparents now live a longer and a healthier life and that our children may enjoy an extended family in the early formative years. It is true that the healthcare system is already experiencing the strain of having more elderly and frail people to care about. It is, however, more accessible and provides more opportunities for diagnosis and treatment than several decades ago. The modern medicine is now rapidly developing and expanding its possibilities in real time. This includes development of tools to assist in earlier identification of people at risk, precise diagnosing and personalised treatment when disease has already developed.

The basic premise of modern healthcare is to make people healthier as they age, so that they may enjoy life (almost) as much as they did when they were younger. It has been always known that some people preserve their physical fitness, their full mental capacity and their positive outlook on life well into advanced age, and that sometimes this ran in families. Such people are commonly referred to as 'ageing gracefully', or, to use the modern term, 'successfully'. 'Successful' ageing is a relatively recently coined definition that may sound jarringly unusual or controversial at first, as ageing has always been described as anything but 'successful'. According to Erickson's personality theory, later adulthood and subsequent old age is the time of the 'integrity vs. despair' crisis, when the final developmental task of retrospection and assessment of one's lifelong achievements and/or failures takes place, as well as dealing with loss and general preparation for death [1278]. If we look carefully at any modern society though, we would not find many healthy older adults that are content with peaceful contemplation of their past lives only, even when they had chosen to retire from work. A significant proportion of people remain active physically and mentally after 65, contributing to their families, their jobs and the society in a fulfilling and unique way. Improved healthcare, higher general level of education, the opportunity for lifelong learning and the increased ages of marriage and childbirth have contributed greatly to this tendency. People of today usually choose to marry and have children after they have completed their education and have attained a level in their careers that allows them for some long-term planning. This means that instead of starting a family in their early 20-ties, as was common several decades ago, now it is quite normal to delay this until the 30-ties or even the 40-ties, provided that medicine may provide assistance in conception and carrying to term of healthy children.

The standard definition of 'successful ageing' is quite straightforward and is usually defined as '*retaining the ability to function independently in advanced age*' [1279-1281]. It has been also been defined as '*multidimensional process encompassing the avoidance of disease and disability, the maintenance of high physical and cognitive function, and sustained engagement in social and productive activities*' [1280,1282]. Understandably, the parameters of these descriptions would be differentially prioritised and/or achieved in different people, and the need for assistance would increase with progressing age.

Successful ageing has always been recognised, respected, and, often, revered, throughout human history. Many records of notable examples exist. Michelangelo finished his larger-than-life Pietà Bandini at the age of 78 and would have probably finished his Pietà Rondanini, had he not made that horseback riding promenade in the rain after a day's hard work at the age of 88 [1283]. Lorenzo da Ponte, the famous opera librettist and poet who

wrote for over 10 composers, produced the first New York public performance of Mozart's Don Giovanni in 1826, at the age of 77 (over three decades after Mozart himself had died – at the age of 35) and published his 'Versi composti' at the age of 83 [1284]. James Watson, one of the famous duo that gave us the double helix model of the structure of DNA published his "Avoid boring people: (Lessons from a Life in Science)" in 2007, at the age of 79. The list of people who created truly spectacular works in their advanced age is much longer, of course, these are just single examples. In any case, old age is clearly not necessarily the age of regret and disability; it could be productive and gratifying to the ageing persons, their families and the society.

The average lifespan of humans has extended beyond middle-to-late adulthood in the last 100–150 years, mainly because of the successes in prevention and treatment of infectious disease and the development of efficient anti-inflammatory therapies. Today, more people live to ages of >80 in (relative) health than before and have the chance for 'ageing gracefully'. This is, however, not always a simple matter of 'living healthy'. Studies of successful agers from all over the world typically bring forth the presence of apparently 'unhealthy' lifelong habits and happy-go-lucky philosophy in health matters at rates practically indistinguishable from the rates in the general population. The search for the explanation of the discrepancy between the rates of conscious attempts at maintaining a 'healthy' lifestyle and rates of 'successful ageing'/longevity has only begun, and science and medicine are still far from understanding all the factors in successful ageing. As this is an area with a rich potential for abuse and fraud, below we will attempt to demystify some of the most popular misconceptions about 'healthy living' and elicit several points that had been proven to play a role in successful ageing.

7.8. Eat right, exercise regularly, die anyway – do popular health misconceptions have any scientific basis at all?

Nothing is sure for me but what's uncertain:
Obscure, whatever is plainly clear to see:
I've no doubt, except of everything certain:
Science is what happens accidentally.
F. Villon, Du concours de Blois (circa 1457).
Translated by A. S. Kline

Popular literature and media are full of advice on 'healthy eating' and 'healthy living'. These sources, however, are not very reliable. Most of the content is copied and pasted from a limited number of sources. The latter means that any error that may have occurred in the initial content is multiplied and circulated. A prominent example is the myth about the iron content in spinach which turned out to be a product of a botched experiment (for more details, see Sutton's 2010 paper in the Internet Journal of Criminology, accessible online from Sutton – Spinach, Iron and Popeye).

There are several issues that interfere with the correct presentation of research facts and, specifically, health advice in popular media. One of the main issues is the one-dimensionality of the presentation of a health problem in media. Unfortunately, this is rarely unintentional, as the target user is easily manipulated by exaggeration of some sides of the problem, neglecting other sides that may be just as important. Non-specialists

normally have trouble with reviewing and appraising multiple sides to a problem when it is outside their area of expertise. Internal controversy often exists between the presentation of the different sides of a problem in media, but it is not easily spotted on a superficial level. The different sides are often separated from one another and treated as completely different entities for a purpose (for details, see below).

The probabilistic approach also presents considerable difficulties. For example, the phrase '*the factor X is associated with twofold elevation of the risk for cancer*' would sound scary to anyone. The authors of generalisations such as these, however, almost never mention the simple fact that there are many type of cancer, some of which are rarely seen in the general population and other that are much more common. In the former case, a twofold elevation is actually not that much higher when assessing individual risk (e.g. 1:1000 vs. 1:500), in the latter, it may make quite a significant difference (1:10 vs. 1:5). Also, it is hard to explain that having the predisposition to develop a disease or condition does not mean that it would develop with 100% certainty. The concept with which the most people struggle is, however, that no 'testing for all diseases' is possible. Many are disappointed that after all the lengthy and expensive testing they have gone through, something unforeseen might occur. This increases the mistrust in the healthcare system and the risk that people would believe simpler explanations telling them what they would like to hear instead of what is true or is likely to be true. Unfortunately, most of the journalists that are responsible for the presentation of the scientific facts to the general public have experience the same difficulties in reviewing and summarising information that is unfamiliar to them. It is, after all, much easier to copy a popular health fad from elsewhere without questioning its validity than to track down research reports supported by experimental data. The general public usually gets is a garbled 'adapted' version written by someone who has very little factual knowledge about the subject matter. Occasionally, health advice in popular media may be close to the scientific truth, but the possible outcomes are often exaggerated and the sources of the information are almost never correctly referenced. There is also the matter of competing interests in the area (specifically, in pharmaceutics and reparative and regenerative medicine). Pharmaceutical companies may assist in the popularisation of health misconceptions in order to identify potential clients and to induce them to buy specific products.

Currently, a plethora of common misconceptions about healthy living are in circulation. Unsurprisingly, most of these are related to risk of various diseases, ageing and longevity. Here, we will only mention a few of the most popular in order to demonstrate that sometimes what everyone says is not the truth.

As UV was mentioned throughout this monograph as a major factor in the pathogenesis of skin cancer, it would be our first example for the one-sided approach to health issues in media. It is known that UV may trigger skin damage and cancer. The latter is a constant refrain in the media in the months of May to September. Indeed, staying in the sun without any protection is risky at best and may be described as 'unhealthy'. At the same time, however, modern visual media promotes unreal body image that is hardly compatible with the idea of healthy living, but is nevertheless advertised as highly desirable. This includes not only skeletally thin models, but also deeply tanned bodies which are commonly used to

represent the peak of health and fitness. Deep skin tan in Caucasians is actually a sign of serious UV-induced skin damage, and, therefore, could hardly be 'healthy'. The other extreme, however – avoiding sun exposure altogether – may also be unhealthy, actually, as UV-induced conversion is the main mechanism for production of the active form of vitamin D3 (cholecalciferol) from the inactive metabolite 7-dehydrocholesterol (vitamin D2) in higher animals and man. This occurs in the deep layers of the skin, as the UV only penetrates several millimetres within the body [117,1285]. Deficiency of vitamin D3 in man may be associated with a variety of health issues – proneness to cardiovascular disease, depression, decreased leptin levels, dysregulation of bone metabolism, immune disorders (specifically, autoimmunity) and cancer [reviewed in 1286-1290]. True, lack of vitamin D3 may be compensated by alimentary intake of the deficient compound, but unless the case is very specific (extreme sun sensitivity, etc.), small doses of UV are undoubtedly necessary for human health and well-being [1291]. It has been known for decades that exposure to sunlight may improve the mood and energy levels. The phenomenon has been shown to be at least partially linked to an elevation of the levels of endogenous serotonin in response to UV-A [1292]. Insufficient daylight (and, probably, UV) exposure has been implicated in the pathogenesis of seasonal affective disorder, as well as in other mental disorders (predominantly related to mood regulation).

Another example is obesity. Obesity (defined as body mass index (BMI) >25kg/m^2) has been universally recognised as a risk factor for development of many diseases and conditions – e.g. diabetes, atherosclerosis, cardiovascular disease, infertility, even cancer. It was mentioned earlier that the percentage of obese adults >50 years of age may be high as 35% and obesity in children may exceed over 15% in some developed countries. Raising the public awareness about the adverse consequences of obesity is recognised as very important in the strategy for minimisation of health impact of obesity. However, the misconception that being thin is actually good for you and that 'you can't be too thin' has, in turn, become a serious issue in the last several decades. The problem is especially common in members of groups in which low body weight may be desired and/or is a mandatory requirement (models, dancers, gymnasts, etc.). The percentage of eating disorders may reach 10% in these populations. Being underweight (BMI <18.5kg/m^2) greatly increases the risk for anemia, osteoporosis, cardiac rhythm anomalies, infertility, and many others. The Western ideal body image is, therefore, in obvious need of revision. What is more, being slightly (but only slightly) above the ideal weight for height and body frame was found to be actually beneficial. More specifically, it is known from clinical practice that in some diseases and conditions the individuals that are somewhat heavier than the ideal weight may have better prognosis compared to those that are underweight or even those with normal weight. For example, in patients with chronic renal failure of non-diabetic genesis, being above the normal weight is a predictor for ***decreased*** risk for development of some potentially dangerous cardiovascular complications. Specifically, the arterial blood pressure is lower and vascular incidents are less frequent in the patients that are mildly overweight than in those with low or normal body weight [1293,1294]. Of course, this does not mean that extra weight protects universally from hypertension or cardiovascular incidents. On the contrary, the risk for healthy obese patients to develop renal failure is higher than for

healthy individuals with normal weight. The pattern described above is valid only for select groups – namely, those with chronic renal failure with genesis other than diabetic, and is by no means applicable to everyone. Similar 'paradoxical' phenomenon is described in patients with atrial fibrillation (a dangerous, potentially fatal disorder of cardiac rhythm). The risk for occurrence of fibrillation is higher for overweight individuals without history of episodes of fibrillation than in individuals with normal weight, but in individuals that already have experienced one episode of atrial fibrillation, the chance for survival is higher for mildly overweight individuals than for those with normal weight or those that are underweight [1294,1296]. A somewhat similar effect is observed with regard to potential toxic effects of anticancer therapy in patients that are slightly above the calculated 'ideal' weight.

Of course, one could argue that the positive effects that are sometimes seen in mildly overweight patients is because patients in poorer general condition are usually under the ideal weight for their height and frame. Thus, it is likely that they would succumb more easily to any type of complications. Definite proof, however, has not been found yet. Of course, nobody can deny that being overweight may be associated with major health trouble, but, apparently, in some cases, it may actually do you good to be slightly overweight. In healthy older persons, the risk for iron deficiency anemia and fractures related to decreased bone density is lower in individuals with body weight at the upper limit of their ideal body weight or slightly above it [1297-1299].

While we are on the subject of misconceptions about healthy eating, there was (and still is) a popular obsession with dieting or fasting as a cure for all diseases or for the purposes of 'detoxification'. Indeed, it was definitely proven in animal models that a decrease of the caloric intake to 70–80% of the average normative amount for age and size of the animals prolongs their life and delays the onset of some diseases associated with ageing [1300,1301]. Analyses of the existing literature revealed that decrease in the caloric intake to 70–90% of the normative amount may decrease the risk for development of some tumours in humans but could not modify the course of ongoing neoplastic disease [1302,1303]. Therefore, fasting cannot cure pre-existing cancer.

The protective effect of reduction of caloric intake may be explained by decrease of the levels of oxidative stress in the cell when smaller amounts of calories are consumed (as there is smaller amount of substrate available for oxidation). Humans, however, are not lab mice that may be kept, exercised and fed according to schedule. Different people may have very different requirements to caloric intake, depending not only on age, height and frame, but also on lifestyle (sedentary, moderately active, very active), hormonal status, levels of stress, and many other factors. Adopting a lifestyle of continuous or periodic semi-starvation, or fasting for days and weeks on end may have very serious short-term as well as long-term consequences. These may be of critical importance in childhood and adolescence. Some of the short-term consequences may be: dry skin and brittle hair, gastroenterocolitis, liver disease, poor mineralisation of bones and teeth, associated with increased risk for fractures and dental and gum disease; growth retardation (in the young); and others. Among the major long-term consequences of inadequate caloric and/or nutrient intake in childhood and adolescence may be – surprising as it may seem– atherosclerosis and cancer. Studies among survivors of wars and natural disasters which

caused long-lasting shortages of food (the siege of Leningrad during the World War II, 'The Hunger Winter' in the Netherlands in 1994 and the Great Famine in China (1959–61) showed that the prevalence of cancer in the survivors, especially those who were children or adolescents during the famine period, was significantly increased (2–3 times) compared to control age-matched populations that had never experienced periods of significant caloric deprivation or starvation [1304,1305]. The same phenomenon was observed for diabetes type 2, arterial hypertension, atherosclerosis and cardiovascular disease [1306-1308]. There is significant amount of research data showing that caloric deprivation or starvation in youth may constitute a serious risk factor for obesity in adults [1309]. Consequences of periodic starvation or systemic underfeeding of pregnant mothers may increase the risk for their unborn children for various diseases in later life, possibly via epigenetic mechanisms [reviewed in 1310]. For example, caloric deprivation during intrauterine life ('hungry in the womb') and early childhood was shown to be associated with increased risk for development of affective disorders, schizophrenia and various substance addictions in later life [1310-1312]. Apparently, neither extreme is a good option when it comes to healthy eating, and popular diet fads may harm not only the individuals practising them, but also their children.

Another interesting example of misleading health advice is the alleged 'harm' of some foods and beverages. The type of 'harmful' foods varies greatly, with some things being bad for you now and good for you several months later. The 'badness' is sometimes too vaguely defined, or is justified by circular reasoning of the type 'it is bad for you because it is bad' (e.g. drinking coffee may be harmful because it bad for your heart). In some cases even data from correctly performed experiments may be distorted in a manner which makes it usable 'for any purpose' – usually, by withholding or manipulating important information so as to bring specific aspects into sharper relief. For example, there are many widely publicised (for lack of a better word) documents about the alleged mutagenic activity of coffee (usually presented in media as 'risk for cancer'). This type of papers usually mentions the Ames test for mutagenic properties [1313]. Indeed, there had been extensive research on the mutagenic properties of compounds in instant coffee and coffee brewed from baked and ground coffee beans [1314-1316]. The results of the studies showed that the mutagenic properties of coffee-based beverages determined by the Ames test were strongly dependent on the manner of processing of raw coffee beans (e.g. roasting). The test was, however, carried out in a prokaryotic model system and the results could not be interpreted directly to reflect the effects of human consumption of coffee. The latter is, however, never mentioned in the 'coffee-induced cancer' publications in popular media.

It has been repeatedly demonstrated that polyphenols and caffeine in tea (specifically, green tea) and coffee have potent antioxidant properties [1317,1318]. 'Crude' (non-purified) caffeine as well as other components in coffee may actually reduce the degree of memory impairment and the level of amyloid in mouse models of Alzheimer's disease [1319,1320]. Caffeine has been shown to arrest (or, at least, significantly delay) the progression of mild cognitive decline to dementia in human patients [1321]. This is not surprising, as oxidative stress plays a crucial role in the pathogenesis of Alzheimer's disease.

Antioxidants have been in the focus of the public attention for some time, especially after the role of oxidative stress in ageing has been demonstrated. They have been widely advertised as prevention and/or cure for all diseases. Again, this exploits the trust of the end user that strives for a 'healthy' lifestyle. A roaring trade in 'dietary supplements', 'micronutrients' and 'antioxidants' has been going on for several decades, starting with megadoses of vitamins (usually, vitamin C) and ending with 'herbal remedies' of unknown composition. Pregnant women are especially vulnerable to this type of advice, as they are often advised to take vitamin supplements. It was reported, however, that 10% pharmacies and over 20% of health retailers may offer advice consistent with vitamin overdose [1322]. The basic concept that is typically sold to the unwary user is that the consumption of foods that have been heavily processed and stored for long periods of time results in deficiency of crucially important biologically active compounds. These compounds ought to have been supplied with food, but were unfortunately destroyed by thermal or other type of processing. Usually, when the potential client asks what these compounds actually were, the typical reply contains any combination of: 'vitamins'; 'minerals', 'microelements'; micronutrients', 'supplements', etc. Of course, if we stop and think about it, we will quickly spot the controversy in such statements. 'Minerals' or 'microelements' are usually simple cations (metal, transition metal or polyatomic nonmetal – calcium, zinc, copper, molybdenum, selenium, etc.) within simple inorganic compounds (chlorides, sulphates, iodides, etc.). Being ions of chemical elements, these compounds cannot possibly be 'destroyed' by heating at the temperature of boiling water (even for prolonged periods of time) or any other type of conventional processing. As a matter of fact, a recent study showed that household cooking actually enhanced the antitumour properties of curcumin, as cooking-induced pyrolysis produced a more active compound – "curcumin deketene" [1323]. As for the vitamins, it has been known for quite a long time that some of them may be degraded by boiling and preservation. The process, however, is very rarely complete within normal cooking time and is strongly dependent on the amount of water used to boil the food, the actual duration of the boiling (hence, the rather sensible advice to cook vegetables 'al dente') and the pH (for example, vitamin C is best preserved in acidic environments).

Usually, when the advantages of vitamin supplements are advertised, the information about possible discomfort and even potential dangers associated with megadose vitamin regiments are withheld from the user. This may occur inadvertently (the seller does not actually know about the possible adverse effects) or deliberately, with the former occurring more often than the latter. For example, use of large doses of Vitamin C is very commonly advertised. The users are rarely informed, however, that taking over 1–2 g of vitamin C per day may cause profuse diarrhoea. In people predisposed to calcium oxalate crystal formation (may be subclinical, even completely asymptomatic), excessive intake of Vitamin C may cause accumulation of oxalate deposits in the kidneys and the bladder. In individuals with glucose-6-phosphate dehydrogenase deficiency, a very common inherited disorder (the prevalence is about 1:20 people, even about 1:4 in some populations), large doses of vitamin C may provoke severe, potentially fatal haemolytic crisis. The user is also typically unaware that taking vitamin C-containing preparations may compromise the results of

some medical tests – for example, presence of ascorbic acid and its derivatives in urine (most of the ingested extra amount is excreted via the kidneys) may cause false positive results in urine tests for reducing substances (glucose – for example the standard urine dipstick test, lactose, pentoses, etc.).

Overdosing on vitamin C is actually quite harmless, especially when compared to excessive use of lipid-soluble vitamins such as vitamin A and derivatives; and vitamin D3. Carotenoids (precursors to Vitamin A) have been widely proclaimed in the last years as anticancer agents and consumption of carrot-based juices and vitamin-fortified juices was strongly advised. Physiological activity of most carotenoids, however, has not been proven experimentally yet, except for carotene (provitamin A) itself [1324,1325]. The user is typically uninformed, however, that excessive use of carrot juice may cause yellowish pigmentation of the skin and, in severe cases, liver damage. What is more, several trials have already suggested an ***increased*** risk of lung cancer for high-risk individuals (e.g. smokers) associated with ingestion of high doses of β-carotene (alone or taken together with other compounds with antioxidant properties) [reviewed in 1326]. Much more solid evidence may be needed before the claims about the beneficial effects of ingesting high doses of non-provitamin A carotenoids are confirmed.

Large doses of derivatives of retinoic acid may be associated with changes in hair colour and texture and hepatotoxicity [1327,1328]. Pregnant women must be especially wary of taking retinoids beyond the recommended daily allowance (RDA), as the latter are known to induce differentiation in undifferentiated cells and may cause inborn anomalies and/or developmental delay in the child [1329]. The latter is the main reason for advising against getting pregnant during treatments with topical preparations including isotretinoin (Accutane, for treatment of recalcitrant acne) or etretinate (Tegason, for topical treatment in psoriasis) and in the post-treatment period (up to 2 years after discontinuation of the use of the retinoid) [1330,1331.

Vitamin D3 is often included in preparations containing calcium, which is typically justified by stating that vitamin D3 'assisted the absorption of calcium'. Indeed, as was noted above, the deficiency of vitamin D3 has been shown to be associated with various pathological phenotypes. Ingestion of extra amounts of vitamin D3 alone or in combination with other lipid-soluble vitamins, however, may cause hypercalcemia and renal failure [1332,1333]. It has been found that hypervitaminosis D3 in mice may induce premature ageing phenotype [1334,1335]. Therefore, it might be wiser not to meddle with vitamin D3-associated metabolism without sound medical advice.

There are, nevertheless, many compounds in foods and beverages that have been shown to have beneficial effects on human health without significant associated toxicity. Such are, for example, resveratrol in red grapes (antioxidant and immunomodulating properties) [1336,1337]; antocyanins, lycopene and other carotenoids in red in red- and yellow-coloured vegetables (antioxidant properties) [1338,1340]; sulphoraphane and indole-3-carbinol in cruciferous vegetables (anticancer effect) [1341], and others. All these fruits and vegetables are components of the normal human diet (or, at least, ought to be). Their beneficial effects, however, are based on prevention (decreasing risks) rather than cure of already developed diseases and conditions. Therefore, there is not much chance that a pre-

existing tumour might shrink if the patient follows a diet based exclusively on radish; or that atherosclerosis might be cured by consumption of red and yellow fruit and vegetables. Including these foods in the everyday diet would undoubtedly be beneficial, but then consumption of fresh fruit and vegetables has been known to be 'good for you' ever since James Lind published his 'Treatise of Scurvy' (1753) [reviewed in 1342].

Some natural compounds (emodin, curcumin and others) have been found to have true anticancer effects, even when the tumour has already developed [1343-1345]. When used as adjuvant agents, these two enhance the anticancer properties of cisplatin via downregulation of the expression of ERCC1 (a NER protein) and thymidine phosphorylase, a key enzyme in the pyrimidine nucleoside metabolism [1346,1347]. These experiments have been carried out in vitro (in cultured human cancer cells) and in vivo (in murine models), but has not been tested on human cancer patients yet.

Whatever the popular literature and media might say, a 'magic bullet' for all age-related diseases and conditions (specifically, for cancer) is not likely to be ever invented. Similarly, a healthy lifestyle cannot possibly ensure 100% prevention against all diseases. Dietary supplements and 'healthy living' have always had their fans, but it would be advisable that the potential users were informed about what they are buying before they actually bought it.

Some of the commonly offered advice about 'living healthy' may be actually theoretically sound and supported by reliable experimental data. However, it is not to be taken as 100% guarantee that following it would ensure health and longevity or, alternatively, that deviating from it would mean imminent disease. Such advice may be, for example: maintaining low total cholesterol levels; prevention of hypercoagulability ('an aspirin a day'); abstaining from drinking alcohol; physical exercise, etc. Extensive surveys among people who qualify for the 'extreme longevity' category (≥85 at the time of the interview), show, however, that the distribution of behaviour patterns (including habits related to personal health) among these oldest old does not differ significantly from those seen in the general population [1348,1349]. We will give a few examples here in order to demonstrate that what is 'healthy' and what is 'unhealthy' cannot always be placed into clear-cut categories.

Many studies have elicited an inverse relationship between regular exercise and the risk of certain cancers [1350]. However, a differentiation ought to be made between physical activity that is physiologically adequate (with regard to gender, age and physical fitness of the individual) and physical exercise that may be inappropriate (e.g. too strenuous). It has been repeatedly shown (in animal models and in humans) that intense physical exercise may be accompanied by increase in the levels of oxidative stress not only in the working muscles, but in other tissues and organs as well [1351,1352]. Oxidative stress is now recognised as a major factor in carcinogenesis; therefore, it is possible that too much exercise may produce the opposite of the health advantages associated with moderate exercise.

It has been demonstrated that the relative risks for coronary heart disease and stroke (typical age-associated conditions) may actually be reduced by consumption of alcoholic beverages in moderation. In other words, the prevalence of cardiovascular disease and

stroke was found to be actually *higher* in non-drinkers than in moderate drinkers [1353,1354]. Lately, it has been demonstrated that drinking in moderation may reduce the risk for mild cognitive decline and/or dementia in people of middle and advanced age [1355,1356]. The line between alcohol use and alcohol abuse is very thin and it is, therefore, advisable not to drink at all, so as to prevent development of alcohol dependence. However, total abstinence from alcohol is apparently another 'healthy living' concept calling for a careful evaluation.

Aspirin daily in low doses has been shown to have a protective effect against vascular incidents – specifically, it decreases the risk of ischemic stroke in women and myocardial infarction in men [1357,1361]. This is valid, however, only when aspirin is used as an adjunct in management of other cardiovascular risk factors, and not as an alternative. 5–25% of the population, however, is resistant to the antiaggregant effects of aspirin in low doses, and high doses may be associated with other type of complications [1360,1361]. Therefore, taking aspirin is not a universal option for all ageing people.

Hypercoagulability was also found to be compatible with successful ageing and longevity [1362-1364]. Specifically, they found heightened coagulation enzyme activity (von Willebrand's factor (VWF), Factor VIII) and enhanced formation of fibrin in the very elderly compared to younger controls. Some of the common prothrombotic mutations (the G20210A mutation in the prothrombin gene, the Factor V Leiden mutation and the 4G allele of the *PAI-1 4G/5G* polymorphism were found to be more common in centenarians than in younger controls [1363].

Total cholesterol levels over 5mmol/l in the age group over 70 years were found to be positively associated with survival due to lower mortality from cancer and infections [1365,1366]. In the oldest old, each 1mmol/L increase in total cholesterol corresponded to a 15% decrease in mortality [1367]. This was rather a surprising finding, as it is known that hypercholesterolemia contributes to morbidity and mortality in young age and adulthood by increasing the risk for development of vascular disease. The phenomenon of an apparently 'harmful' factor being common among the oldest old may be explained by 'reverse' antagonistic pleiotropy. Pronounced prothrombotic tendencies and/or elevated cholesterol levels may be disadvantageous in young age by increasing the risk for cardiovascular disease and/or vascular incidents, but in later age they may turn out to be more of a selective advantage than a disadvantage by decreasing the risk for other common causes of mortality. The exact mechanisms are, as of now, unknown.

In 2003 were published the results from a large study of over 400 individuals aged 97–119 years, that stated that morbidity profiles of centenarians generally fit into one of three large categories – 'survivors', 'delayers', and 'escapers' [1368]. 'Survivors' were these that had been diagnosed with age-associated degenerative disease or cancer before the age of 80. 'Delayers' were these that developed age-associated disease after 80, and 'escapers' were those that remained essentially disease-free up to the age of 100 or more. The proportions of the different categories in the overall morbidity profile were different, but not drastically different, and in some categories there was marked differentiation by gender. Specifically, 24% of male subjects and 43% of female subjects were classed into the 'survivor' category; 44% of the male and 42% of the female subjects fit the 'delayer' category, and 32% of male

and 15% of female subjects fit the 'escaper' category. The gender differences were not surprising, as it has been known for a long time that women typically live longer than men, but had more health conditions compared to age-matched men. Analysis of morbidity for the most lethal diseases of the elderly population – heart disease, stroke, and non-skin cancer showed that over 80% of all centenarians in the study (87% of the men and 83% of the women) had delayed onset of these diseases or had escaped them altogether in their old age. Recently, in 2012, 'the compression of morbidity' hypothesis was formulated, that proposed that people who live to >107 years of age tend to 'compress' morbidity and disability in the very ends of their lives – that is, they are generally healthy throughout their very long lives and develop serious diseases only at very advanced age [1239,1369]. We already saw, however, that there may be people living to >95 in all morbidity categories, and that being healthy in youth does not guarantee health in middle and advanced age, therefore, there is no way of telling by the morbidity pattern in young age how the life expectancy is shaping for the particular person, unless there is serious disease developing at young age (e.g. diabetes).

At present, it seems that the optimal approach to management of ageing and age-related diseases is delaying their onset or the development of disease-associated complications for as long as possible. This may be achieved by lifestyle alterations, elimination of some harmful habits and, whenever needed, medication and/or other types of medical treatment. Stress management may also be helpful, as positive outlook on life seems to be a very important factor in successful ageing as well (see below). In any case, however, it is very difficult at present to make a reliable prognosis of the morbidity profile of an individual or to estimate their life expectancy with precision and the only reliable criterion remains the family history of age-related disease and the age of death of relatives from the direct line.

7.9. Could we really do anything to ensure successful ageing?

Let us admit, Doctor, that when young age conspires against old age,
everything the latter does to prevent the former from doing exactly
what it pleases may very well be called a useless precaution
Pierre-Augustin Caron de Beaumarchais, The Barber of Seville,
or The Useless Precaution (1773).

Several properties associated with successful ageing have been defined so far. Among these, two basic categories could be differentiated: traits that are associated with physical status and traits related to personality and general attitude towards life.

Of the first category, some of the general characteristics considered to be related to increased chances for successful ageing are: less health trouble that is typical for people in this age (no diabetes, no cancer, no serious neurological or mental disease); and preserved cognitive and physical capacity (adequate for age). The latter may greatly vary as age advances. For example, in the 'younger' old (75–85) the criterion of 'preserved physical capacity' may mean 'takes regular walks for pleasure and/or exercise', while in the oldest old (>95) this may mean 'capable and/or willing to walk should the need arise', although some of the oldest old may be more active. The same is valid for the cognitive capacity, considering that eyesight, hearing and vestibular sense may grow weaker with age. The

need for assistance typically grows as age advances, but the younger old may be perfectly able to live independently and choose whether to live with other people or not, while the oldest old may need assistance with some aspects of daily care (e.g. bathing, shopping, cleaning the house, preparing food, etc.) and may benefit from living in a shared household. The second category of qualities associated with successful ageing is considered as important as the first. They comprise several traits that are associated with personality characteristics (active engagement in life, satisfaction with life, general optimistic mindset towards life); and one characteristic that is dependent on external factors – namely, one's spouse alive and (relatively) healthy [1370-1373].

A specific lifelong trait associated with longevity and successful ageing is adequate physical activity, both leisure and non-leisure. The beneficial effects of adequate exercise were found to reflect on both components of successful ageing – that is, on physical as well as on psychological well-being [1373-1376].

Both groups of traits characteristic of successful ageing seem to be important and while neither could actually exist without the other; both could exert a synergistic effect towards living a long and full life. The traits associated with successful ageing seem to have a single feature in common – independence, both in physical as well as in psychological aspect. At present, sustained independence as age advances is the single major predictor for ageing gracefully and living beyond the average lifespan of the population that seems to work in real time. Being independent at, for example, age of 75 predicts that there is reasonable chance that the individual is likely to continue being able to live independently several years later. This is actually quite natural, as people who suffer from serious diseases are less likely to live independently and the risk of potential complications increases as age advances. Even in the healthy old, however, maintaining independence is important for the psychological well-being and may boost self-esteem.

Modern medicine offers many opportunities to prevent the risk for development or delay the age of onset of symptoms of many age-related diseases and conditions – for example, early screening for cancer, glucose intolerance, hypertension, etc., or, when the disease or condition have already developed – anticancer therapies and/or surgery, antidiabetic therapy, antihypertensive medication, etc. The positive outlook and genuine interest in life, however, which is the other major factor in successful ageing, is usually dependent on the character and general attitude of the person. Therefore, living healthy is only one component of being healthy and ageing gracefully; living well is the other. The former does not seem to work just as well without the latter.

8. Temporal and spatial distribution of DNA repair

8.1. DNA repair in terminally differentiated cells

It is a painful thing to say to oneself: by choosing one road
I am turning my back on a thousand others.
Antonin-Gilbert Sertillanges, The Intellectual Life:
Its Spirit, Conditions, Methods (1923)

At any given moment, only a very small part of the DNA in eukaryotic genomes is being transcribed. Some genes are switched on at some point in individual development and switched off later, or may be only activated in dividing cells, therefore, the percentage of transcribed DNA may vary in different phases of the life cycle, but would generally not exceed several per cent of the genome throughout the life of the cell or the organism.

We have already discussed the advantages of having the coding DNA sequences buried within a huge amount of 'junk' (non-coding) DNA in order to protect them from the random 'hits' of mutagenesis. Indeed, the risk that a mutation event may affect a crucially important site is much lower if that site is surrounded by many other sites that may also serve as potential mutation targets. There are, however, additional natural mechanisms for protection of coding DNA, complementing the default mechanisms for detection and repair of DNA damage. Specifically, genomic regions that are actively transcribed at any given moment may be repaired with priority to the untranscribed DNA (transcription-coupled repair, TCR, TC-NER) [238,239, reviewed in 1377]. There may be several sub-levels to this prioritisation. The transcribed strand of the gene may be repaired with priority to the untranscribed strand [239,241]. Different exons in the same gene may be repaired more efficiently than others [1378,1379]. It has even been reported that prioritisation may exist even at sequence level (damage at specific nucleotides may be repaired first) [1380-1382].

It is currently believed that the mechanisms for protection of transcribed DNA were put in place to manage the balance between the immediate harmful effects of DNA damage occurring in the transcribed regions and the delayed pro-carcinogenic effects associated with accumulation of errors in the untranscribed regions of DNA. A mutation occurring in a gene undergoing transcription is likely to have immediate harmful effects for the cells, the tissues and the organism. This may happen in all types of cells, regardless of whether they are capable of division or not. Mutations occurring in non-transcribed regions usually cause no change in the phenotype at all, but some mutations may trigger cancer growth. Impairment of the cell's functions may bring the death of the cell and the organism quickly, while cancer may not. Carcinogenesis usually takes years or decades, as it is related to multiplication of errors and introduction of additional mutations by cell division. In cases when the tumour is indolent and/or manifests at advanced age; and/or when efficient treatment is readily available (in modern settings), the affected individual may live about as long as the average individual in the population, or, possibly, even longer. Thus, keeping in check the transcribed regions in DNA provides that the cells implement their specialised functions normally so that that the organism is fit and healthy; while global genome repair ensures that as few as possible mutations are passed on to the progeny.

Mechanisms for protection specifically from DNA damage that may kill the cell here and now would, therefore, be targeted specifically at specialised cells, as during their time as specialised cells their functioning may greatly influence other cells and tissues in the organism (e.g. by producing specific substances, generating and/or relaying specific signals, etc.). Specialised cells are usually regularly replaced (although some may be long-lived), therefore, the loss of a cell may be compensated relatively quickly by stimulating the proliferation of the adult stem cell niche. In rapidly cycling cells, the 'regular' mechanism for repairing DNA damage as it comes may be more important, as all DNA must be checked and

re-checked before replication anyway. Of course, the overall capacity for repair and for cell and tissue renewal would decline in old age, eventually bringing the death of the organism, be it of decreased supply of specialised cells (degenerative disease) or of cancer.

Cell specialisation usually goes hand in hand with cell differentiation, and the terms 'specialised cells' and 'terminally differentiated cells' are typically used as synonyms. The latter is, however, not always correct, as terminally differentiated cells are always specialised, but specialised cells may not be always terminally differentiated. Specialised cells do not normally divide as they are arrested in G0 phase (although there may be exceptions). Terminally differentiated cells may exhibit certain unique properties. For example, they may be supposed to last a lifetime or to be replaced only infrequently. The usual examples are adult CNS neurons and cardiomyocytes. When damaged, they are not replaced quickly (if at all) and the adult stem cells of the tissue (neural stem cells and cardiac progenitor cells, respectively) exhibit very limited capacity to re-colonise damaged tissues and restore the functions that had been lost [1383-1385].

About a decade ago it was revealed that not only the transcribed genes were repaired with priority in living cells, but that the repair in non-transcribed DNA might be actively repressed in terminally differentiated cells. Specifically, in 2000 Nouspikel and Hanawalt published the results of their studies on the DNA repair profile in neurons, reporting that the cells apparently concentrated their repair efforts on transcribed genes only, with only a very small proportion of the overall repair activity targeted at non-transcribed genomic regions. The phenomenon was initially attributed to downregulation (reported to be to 70% of the values measured in the control group) of the expression of XPC and hHR23B, the factors that play a role in the recognition of damage in non-transcribed DNA. No significant decrease was found, however, in the levels of mRNA of neither of the two proteins [1386]. At the time, it was believed that neurons in adult CNS could never be replaced; therefore, it was proposed that the observed phenomenon was probably specific to cells with minimal proliferation capacity [23].

Selective focusing of repair capacity at transcribed genes at the expense of GGR in terminally differentiated cells is believed to be based at least partially on a parsimony principle. As terminally differentiated cells do not usually divide (with some notable exceptions), mutations occurring in the cell's DNA would not be transmitted on to the next generation, as the cell is not expected to produce progeny. In this case, repair of non-transcribed DNA in terminally differentiated cells may be deemed redundant, as the only genes needed for the immediate survival of the cell and the implementation of its functions are promptly repaired and accumulation of mutations over cell divisions is impossible anyway. It was also proposed that global genome repair was carried out with lower efficiency in terminally differentiated cells because of the more difficult access of the cellular machinery for repair to the non-transcribed regions of the genome, related to the tighter packaging of DNA in the heterochromatin. This was supported by the finding that in neurons the untranscribed strand of DNA in a transcribed gene was repaired with relatively high efficiency [1386,1387]. TC-NER is usually triggered by stalled RNA polymerase II at the damage site, but RNA polymerase II has no physical access to the untranscribed strand of transcribed genes. Since the chromatin structure is typically relaxed in the vicinity of a

transcribed gene, it might indeed be the case that the access of the repair machinery to transcribed genes is easier than in non-transcribed DNA. The mechanism of repair of the untranscribed strand of transcribed genes was called DAR (differentiation-associated repair, also transcription domain-associated repair, TDAR) 1387,1388]. It is essentially a subtype of TC-NER that is independent of the presence of stalled RNA polymerase II at damage sites but is dependent on the 'open' conformation of the chromatin in the transcribed region. It is believed that DAR is responsible for the maintenance of the integrity of DNA in actively transcribed genome regions. Therefore, the state of the chromatin at specific sites may be an important factor in the prioritisation what and when to repair (for more information, see below).

Apparently, both hypotheses that were proposed in order to explain the unusual profile of DNA repair in terminally differentiated cells turned out to be valid – that is, they may afford to carry out DNA repair in transcribed genomic regions only because the risk of carcinogenesis associated with persistence of unrepaired damage was negligibly low and because the euchromatin was more permissive to repair than heterochromatin anyway. This, however, is not viewed as a 'line of least resistance' mechanism, but, rather, as an active decision for focusing the capacity of the cellular repair machinery onto DNA damage in the genes that are important for the function of the cell at the moment, instead of diverting significant part of it to checking and repairing damage that is not likely ever to have any consequences for the cell [23,1389,1390]. GGR, however, is not completely inactive even in terminally differentiated cells. Rather, it is actively suppressed to the lowest level possible.

Later, differences in the efficiency of TC-NER and GGR were found in terminally differentiated cells other than neurons [1391,1392]. It was also noted that the type of the cells and the type of the damage might influence the differential rates of repair in transcribed and non-transcribed regions of the genome. Specifically, terminally differentiated rat cardiomyocytes irradiated with UV were shown to efficiently repair 6-4 photoproducts in the non-transcribed regions of the genome, but the rate of repair of thymine dimers in the non-transcribed regions was relatively poor [1391].

8.2. Modulation of efficiency of DNA repair in cell types that are not terminally differentiated

Expense, and great expense, may be
an essential part in true economy.
Edmund Burke, A Letter to a Noble Lord (1796)

Not only terminally differentiated cells are capable of selective downregulation of DNA repair. Other types of non-dividing cells may suppress overall repair, in a passive (e.g. by chromatin compaction) as well as in an active manner. For example, in one of the studies mentioned above, differentiated rat spermatogenic cells exhibited very low levels of overall DNA repair, both in untranscribed genome regions and in transcribed genes [1391]. This was proposed to be related to the chromatin condensation typical of differentiated cells, on the one hand; and targeted sequestering of NER proteins, on the other hand.

Some cells capable of proliferation (e.g. differentiating cells) may maintain high efficiency of their transcription-dependent repair at the expense of suppression of global repair, similarly

to terminally differentiated cells. It was first demonstrated in acute myeloid leukemia cell lines [1393]. There was significant variation in the rates of repair of different types of damage in genomic regions with dissimilar transcription status, with cyclobutane pyrimidine dimers being repaired proficiently in transcribed regions and poorly in non-transcribed regions. Later, repression of global genome repair was observed in circulating quiescent human B-lymphocytes (memory B-cells) [1394]. The latter are normally arrested in G0 phase of the cell cycle and divide only in response to a trigger (usually, a specific antigen). Once activated, however, they proliferate rapidly, mounting a powerful secondary immune response to a known antigen. It has been proposed that the GGR downregulation in memory B-cells may be associated with accumulation of DNA damage and increased risk for carcinogenesis of B-cell origin (B-cell lymphoma) [1394,1395]. Since different B-memory cells are specific to different antigens, however, the risk for accumulation of sufficient number of mutations per cell so as to trigger cancer growth through repeated activation of the same memory cell is quite low.

There might be prioritisation of DNA repair even within specific sub-mechanisms. For example, it has been demonstrated in human cells that different types of DNA damage occurring in transcribed regions were repaired at similar high rates, regardless of the type of the lesions (adducts caused by cisplatin, photoproducts caused by UV light and crosslinks caused by angelicin). At the same time, damage occurring in non-transcribed regions was repaired by GGR with varying efficiency, depending on the type of lesion. This might have been related to differential rates of recognition of lesions, as they were associated with different degrees of helix distortion [1396].

In differentiating cells, the current phase of differentiation may play a role in the efficiency of DNA repair. We already mentioned a study reporting that that overall DNA repair was suppressed in rat spermatogenic cells. Extracts from rat meiotic cells in pachytene, however, were shown to display high NER-associated 'dual incision' activity in vitro [1391]. Dual incision activities of extracts from cells in later stages of spermatogenesis, however, specifically, cells in diplotene and round spermatids were low, as in differentiated cells.

Activation of TC-NER at the expense of GGR is may be not restricted to selected types of cells only. It was already mentioned that GGR in rodents was usually carried out with low efficiency, with practically all repair efforts concentrated at the actively transcribed genomic regions ('the rodent repairadox') [22,239,242]. Rodent cells may survive much larger doses of UV than any other mammalian species without sustaining severe damage. The main reason for that is that almost 100% of the repair activities are concentrated on the actively transcribed genomic regions whereas the remaining part of the genome is also repaired, but at a very low rate. For comparison, healthy human cells would normally attempt to repair all instances of damage, regardless of whether it is located in the transcribed or in the non-transcribed genomic regions. The ratio of the efficiency of repair of the transcribed strand versus the non-transcribed strand of transcribed genes may also show significant differences between the different species – up to 10 in rodents, 5 in yeast and as low as 2 in humans [1397]. Presumably, check-ups of the integrity of the whole genome are carried out in rodent cells only as part of the routine preparations before DNA replication, as it has been reported that cells of rapidly proliferating rodent tissues (e.g. liver, kidney and testis)

show higher capacity for repair by nucleotide excision than cells from slowly proliferating tissues (heart, skeletal muscle and lung) [1398].

Given enough time, human cells would eventually repair all damage to their DNA (admittedly, these in the actively transcribed regions would be repaired with priority), while rodent cells would allow persistence of high levels of unrepaired damage in the non-transcribed regions as long as the transcribed genes are promptly repaired. A possible explanation may be that rodents were naturally programmed for a short lifespan, therefore, they could afford to dispose of global genome repair, while long-lived mammalian species could not, as the latter would greatly increase the risk for carcinogenesis. The risk for accumulation of unrepaired mutations typically increases with the number of cell divisions. The genome size and the rates of spontaneously occurring mutations are very similar between different mammalian species. The incidence of spontaneously arising tumours in rodents is, however, comparable to the incidence in other mammalian species, with the incidence typically rising as a function of age. Mice and rats normally live for only 2–3 years (the threshold of living beyond the average lifespan being set at 800 days) and cultured murine cells only divide about 15 times before reaching the Hayflick's limit [1168]. Therefore, the risk for carcinogenesis because of accumulated damage in DNA in rodents is naturally limited by the short lifespan of the species. For species programmed to live for several decades, however, as humans are, a repair schedule based on focusing on transcribed genes only is not likely to work. There are, however, exceptions to this, as could be seen below, albeit they are very rare indeed.

Sometimes you can eat your cake and have it – the case of the naked mole rat

There is one very notable rodent species that challenges effortlessly all modern theories of aging and carcinogenesis by being exceptionally long-lived (for a rodent) and, at the same time, exceptionally healthy throughout its life. This is the naked mole rat (Heterocephalus glaber – Fig. 25), a small subterranean rodent that may live well up to 30 years.

Figure 25. The most successful ager known to science of today – a female breeding naked mole rat feeding her pups. (Picture from Pearson Education, Inc. (2009).

The biology of H. glaber is highly unusual. It is one of the only two species of mammals known to modern science that are eusocial, that is, they live in structured colonies with complicated hierarchical organisation and intricate schemes for breeding, care of the offspring and distribution of labour between individual members, similar to the organisation of anthills and beehives. Naked mole rats have very high pain threshold, very low basal metabolic rates, use oxygen very sparingly (which makes sense, as they live underground) and are thermoconformers, that is, they do not increase their metabolic rate when the ambient temperature decreases in order to keep warm but, rather, their body temperature may vary according to the changes in the temperature of the environment.

Among their other astonishing features, naked mole rats are exemplary 'successful agers', changing very little from the moment they are full grown to their ripe old age and suffering very little from typical diseases of ageing, such as cardiovascular disease. Breeding females (queens) show no decline in fertility even when they are well beyond their second decade of life [reviewed in detail in 1399]. Spontaneous occurrence of cancer in the naked mole rat has never been noted yet, neither in the individuals living in the wild, nor in those bred in captivity [reviewed in detail in 1399 and 1400]. For comparison, the risk for cancer in mice increases as age advances until approximately day 800 of individual life, then plateaus, and if the mice live beyond 800 days, it may actually decline [1401].

The last common ancestor of mice and mole rats lived approximately 70 million of years ago. Members of a related genus of blind mole rats (Spalax spp.) partially share the characteristics of exceptional longevity and tumour resistance of the naked mole rat, although blind mole rats live up to 20 years only [1402]. Both species exhibit preserved telomerase activity in their somatic cells.

There has been extensive research on the possible causes for the observed phenomenons in the naked mole rat, but the only relevant finding so far was that cultured fibroblasts of H. glaber grew much more slowly in culture than fibroblasts from other rodent species and achieved contact inhibition at densities much lower than normal fibroblasts from any mammalian species [1403]. As the capacity for contact inhibition is typically lost in cancer cells, it is now believed that the exceptional resistance of the naked mole rat to carcinogenesis may be based (at least, partially) on specific mechanisms for inter-cellular signalling.

Genes transcribed by RNA polymerases other than RNA polymerase II may also be repaired with preference, depending on the transcription status of the gene in question. Genes transcribed by RNA polymerase I may also be repaired with priority by the TC-NER mechanism [247,1404]. Genes transcribed by RNA polymerase I (mainly, ribosomal genes) are present in multiple copies per genome, but only a fraction of them is actively transcribed at a given moment. The chromatin in the ribosomal gene loci may exist in different states – "open" state, typical of transcribed genes or a "closed" state, tightly packed in nucleosomes [1405,1406]. In 2012 it was experimentally proven that NER was more efficient in non-packed than in packed ribosomal DNA [246,247]. The non-transcribed strand of genes transcribed by RNA polymerase I is also repaired at a faster rate than non-transcribed DNA [1404].

There seems to be at least one known exception to the rule 'if it is transcribed, it is repaired with priority'. It has been shown that the genes transcribed by RNA polymerase III (coding for tRNAs and other small RNAs, such as 5S ribosomal RNA) were not repaired at a faster rate than the non-transcribed DNA. tRNA genes did not exhibit significant differences in the efficiency of repair of the transcribed and the non-transcribed strand as well [244]. Considering that genes transcribed by polymerase III usually reside in genomic regions that are free of nucleosomes ('open' conformation), the reported slow repair rate of such genes is unusual. Repair of 5S rRNA genes (also transcribed by RNA polymerase III) may exhibit prioritisation of repair sites within the gene, with suppression of repair of cyclobutane pyrimidine dimers within the internal control sequence, while dimers in some sites within the gene were repaired with priority [1407].

Until recently, it had not been clear whether prioritisation of DNA repair with regard to the transcription status of the repaired region occurs in plants. Lately, it has been shown that in Arabidopsis thaliana pyrimidine dimers were removed from the transcribed strand more rapidly than from the non-transcribed strand [1408].

Some types of cells are capable of modulation of the efficiency of repair of their DNA in some phases of the life cycle, then restore it back to normal as they transition to the next phase. For example, mammalian monocytes may actively inhibit some of the basic pathways for repair of DNA damage, at the same time enhancing the apoptosis-associated signalling. As a result, monocytes that have sustained damage may not attempt repairs, but, rather, die rapidly by apoptosis via activation of the ATM/ATR pathway. The suppression of DNA repair is implemented by downregulation of the expression of several key proteins of damage-related signalling and proteins of the excision repair and double-strand break repair pathways – namely, XRCC1, ligase III alpha subunit, poly-(ADP-ribose)-polymerase 1 and the catalytic subunit of DNA-PK [1392]. Monocytes treated with genotoxic agents accumulate double-strand breaks because of deficiency in NHEJ and single-strand breaks because of faulty base excision repair. The recognition and removal of modified nucleotides is intact in monocytes and some enzymatic activities of BER are actually upregulated (e.g. O6-methylguanine-DNA-methyltransferase) [1392]. The deficiency of ligase III and its accessory factor XRCC1, however, results in occurrence of multiple unligated breaks in DNA that serve as a potent pro-apoptotic signal. Monocytes are partially differentiated cells, precursors of two types of mature immunocompetent cells – macrophages and dendritic cells. By the time they have reached monocyte stage, the proliferation programme of the differentiating precursor cell is normally 'locked' and further differentiation occurs without cell division. Upon receiving a differentiation signal (in response to infection, inflammation, etc.) monocytes release various cytokines, attracting other immune cells at the site and modulating the local immune response, and differentiate further into macrophages and dendritic cells. Macrophages destroy their targets (microbial and protozoan agents, infected cells, transformed cells, aged or damaged cells, etc.) by generation of massive amounts of reactive oxygen species ('oxidative burst'). Dendritic cells function as antigen-presenting cells in the sites where the encounter with antigens is more likely, that is, the skin, the epithelium of the respiratory and the gastrointestinal tract, and, in small amounts, may also be present in peripheral blood. The presence of ROS at sites of inflammation stimulates the

maturation of dendritic cells. ROS are, however, quite harmful to normal cells, therefore, the mechanisms of activation and deactivation of ROS-producing immune cells are tightly controlled at several levels and failure of deactivation has been found to be associated with human disease. These are diseases and conditions with significant inflammatory component in their pathogenesis, such as atherosclerosis, rheumatoid arthritis, inflammatory bowel disease, and others [1409]. The lifespan of macrophages and dendritic cells is limited, from several days up to two weeks, and they usually die shortly after the cause of monocyte activation had been eradicated (e.g. the infection had been cleared). The induction of differentiation of monocytes into macrophages and dendritic cells is accompanied by upregulation of the expression of the repair proteins deficient in the monocyte precursor. Differentiated macrophages and dendritic cells are, therefore, repair-proficient [1410]. The incapacitation of several DNA repair pathways in monocytes is believed to be a negative feedback mechanism limiting the production of ROS after the process that had induced the activation of the monocyte precursors was put under control. Thus, the production of ROS-releasing and ROS-modulated cells is controlled at a preliminary stage and may be cut down abruptly when there is no longer need for these cells.

8.3. More 'repairadoxes' – is global genome repair actually disposable in mammals?

The little mouse, how sagacious an animal it is,
which never entrusts its life to one hole only;
inasmuch as, if one hole is blocked up,
it seeks another as a place of refuge.
Titus Maccius Plautus, Truculentus, Act IV, scene 4 (c. 200 BC)

There may be particular features in the distribution of the efficiency of DNA repair that are specific to whole groups of organisms rather than to cell types. The rodent 'repairadox' is only one of the features of DNA repair and genome maintenance that are specific to rodent cells only. For example, in mouse embryonic stem cells (mESC) the restriction checkpoint (R) in the transition between the G1 and S phase is inoperative, that is, mESC that have sustained DNA damage would typically proceed with the cell cycle [1411]. Indeed, some of the damaged cells would be diverted to the differentiation pathway. In differentiated rodent cells the activity of the R checkpoint is restored, therefore, they may induce repair-associated cell cycle arrest or, in case the levels of DNA damage had been assessed to be beyond certain level, the cells may be eliminated by apoptosis [1412,1413]. ESC of other mammalian species, including human ESC, have their R checkpoint functional, albeit not at full capacity. Therefore, in the presence of DNA damage human ESC are normally rerouted directly to the apoptosis pathway. There have been attempts to explain why embryonic cells of species that are so closely related (up to 90% similarity at genome level) might exhibit different DNA damage-associated behaviour, as normally all mammalian embryos develop in a very similar manner at early stages of development. One of these explanations proposes that cells of the early embryo (and embryonic stem cells as their in vitro counterparts) are naturally programmed to operate under the restricted timeframe of early pregnancy, where degrees of freedom are very limited. Damage-directed cell cycle arrest for repair of damage may not be an option in rodent embryonic cells, as it may greatly reduce

the chances for survival of the embryos. In short-lived species, as are most rodent species, missing a generation cycle may be critical. Reprogramming damaged cells to differentiation increases the chances for embryo survival but, at the same time, carries an inherent risk of transmitting potentially harmful mutations to the progeny [1414]. If the rodent embryo survives under these conditions, the adult organism would rely mainly on TC-NER to repair DNA damage and mutations would continue to accumulate, but the genetic programming of the species would normally eliminate the individuals by 'death of old age' beyond the critical limit of 2–3 years. In species with natural lifespan exceeding several years, however, as humans normally are, even if embryos that have sustained DNA damage survive the early intrauterine development, they would be at unacceptably high risk of cancer from their very beginning, unless development is arrested until the repairs are complete. The latter is hardly an option, however, as early intrauterine development follows a very tight schedule, with many successive phases, and failure to complete the previous phase usually results in death of the embryo. Thus, there is actually very little choice between attempting cell cycle arrest for repairs in the early embryo and reverting directly to programmed cell death, as the damaged embryos are likely to die anyway.

Apparently, GGR is at least partially 'disposable' in higher eukaryotes, but is impractical in organisms with average lifespan exceeding several years. In most long-lived species, only the cells that are not likely ever to divide – that is, terminally differentiated cells or other specialised types of cells – may be allowed to skip repair of non-transcribed genomic regions [1390]. In the rare cases when GGR is incapacitated in man (e.g. because of inherited defects in genes coding for products acting specifically in GGR (XPC) or in later stages of NER (other XP proteins), the risk for cancer related to unrepaired damage increases rapidly after the first years of life. Diseases associated with loss of the capacity for global genome repair are, however, compatible with independent life (albeit in a protected environment) and seldom affect organs and systems other than the skin. For comparison, defects in genes coding for products functioning specifically in TC-NER (e.g. CSA, CSB) usually produce a severe phenotype affecting multiple organs and systems (Cockayne syndrome). Only about 20–30% of children with XP exhibit neurological involvement and its severity may significantly vary, depending on the affected gene. The phenotype of Cockayne syndrome, however, always comprises severe neurological abnormalities. Mutations in genes encoding proteins acting downstream of the step in which the initial recognition of the damage takes place (that is, the essential difference between TC-NER and GGR) usually result in severe phenotypes of XP, mixed XP/CS or trichotiodystrophy, in which neurological deficits are almost always part of the clinical picture.

The tissues that are likely to suffer the most from deficits in GGR are the tissues with rapid turnover – skin and mucosa, and, probably, endothelial and blood forming tissue. Defects in the XPC gene, coding for a protein that plays a role in global genome repair only, produces a phenotype characterised exclusively by proneness to cancers of the exposed areas of the skin and mucosa (XP-C). The incidence of tumours other than skin cancer in XP-C is reportedly very similar to the rates in the general population. Neurological manifestations are unusual in XP-C, to the point that their development may constitute a basis for reviewing the diagnosis. Considering the physiological suppression of GGR in terminally

differentiated neurons, this makes sense, as they would not be affected at all or affected only mildly by the underlying molecular defect.

Cockayne syndrome and trichotiodystrophy are not generally associated with cancer-proneness, unless it is within a mixed phenotype of XP-CS. In the latter cases, as the underlying mutation results in production of a protein that is dysfunctional both in TC-NER and GGR, increased propensity for development of cancers could be expected. If, however, the mutation results in impairment of TC-NER only, it is likely to interfere directly with the functioning of the cells and cause the phenotype of accelerated ageing and/or apoptosis typical of Cockayne syndrome, but would be unlikely to unleash cancer growth.

Cockayne syndrome may become manifest very early in individual life (may even be present at birth). The life expectancy of individuals with both types of CS, XP-A and the phenotypes associated with defects in the *XPB* and *XPD* genes is low, because of the cancer-proneness, the accompanying multiple deficits, and, in the case of mutations in the *XPA* and *CS* genes, possibly the presence of inborn anomalies. The onset of symptomatic disease may be delayed for years in the babies affected by XP-C, although extreme sun sensitivity may be noted from very early age. Generally, in individuals with XP-C, however, provided that adequate UV protection, monitoring and treatment are available, the individuals may live well into adulthood, receive normal education (in a UV-protected environment) and have a job (again, in UV-shielded work settings). This supports the concept that resorting to TC-NER may be a temporary solution that would only work in organisms with naturally limited lifespan.

Surprising as it may seem, deficits in global genome repair in human CNS neurons may play a role in advanced age, specifically in the rare cases when the cells may be forced to re-enter the cell cycle. Neurogenesis in the adult central nervous system was considered impossible up to 1998, when it was demonstrated that new neurons were actually produced in adult brains [1415]. Several years later, however, it was still believed to be a rare event, until in 2004 it was reported that olfactory receptor neurons in mammals were actually replaced every 6 weeks or so throughout the life of the individual [1385]. About the same time (2003) was obtained the first proof that repressed global genome repair in neurons could be a pathogenetic factor in some of the typical diseases of ageing. Specifically, it was demonstrated that unrepaired damage in the non-transcribed regions in neurons played a role in the pathogenesis of Alzheimer's disease [1416]. Brains of patients with Alzheimer's disease typically show neuronal apoptosis and tissue loss throughout the brain. Early Alzheimer's disease is characterised by previously quiescent neurons expressing markers characteristic of attempted re-entry into the cell cycle, such as CDK2 and CDK4, as well as inhibitors of cyclin-dependent kinases, such as p16 [1417,1418]. These are, however, typically failed attempts, resulting invariably in cell death by apoptosis [1419,1420]. It is now believed that the mass programmed cell death in neurons of patients with Alzheimer's disease is related to accumulation of unrepaired lesions in genes that have been switched off for decades, then back on (e.g. genes coding for positive regulators of cell proliferation) [1416,1421].

8.4. Role of DNA repair in the maintenance and the dynamics of chromatin structure

It has been proposed quite a long time ago that the differential rates of repair in various regions of the genome were related to chromatin topology and real-time chromatin remodelling. Higher-order organisation of DNA, including nucleosomal packaging are likely to present steric hindrances to the access of repair machinery to sites of damage. This may be valid for virtually all types of DNA repair, except, perhaps, strand breaks, as the presence of the break would cause partial disruption of higher-order structures at sites of damage.

By the late 80-ties of the XX century, it was already known that damage in DNA from the internucleosomal linker was repaired by NER with priority compared to damage occurring in DNA wrapped around the nucleosomal core [1422,1423,reviewed in 1424]. As prioritisation of repair in different genomic regions has been attributed predominantly to NER, it was quite surprising when it was discovered that some of the glycosylases of BER as well as DNA polymerase β (functioning in the gap-filling step in repair of naturally occurring abasic sites, uracil in DNA, and single-strand breaks) may exhibit differential efficiency in repair of damage occurring at sites with dissimilar nucleosomal organisation [1425,1426].

In 1989, Terleth et al. demonstrated that the differential rates in the repair of the homologous loci HMLα and MATα in yeast were related to their chromatin organisation. The MAT locus in yeast contains the active mating type 'cassette' that is currently being expressed. The HML (Hidden Mat Left) locus contains a copy of one of the 'silent' cassettes, determining one of the mating types (α), while HMR (Hidden Mat right) silent cassette contains a copy, specific for the opposite mating type (the "a" allele). The mating type switch usually occurs when a copy of the 'silent' cassettes replaces the active cassette by recombination by the 'copy and paste' mechanism. Usually, the new copy is of type opposite to the copy that was active to the moment of switching. The active MATa locus was found to be repaired preferentially to the inactive HMLa, although the sequence in both loci was the same. In a mutant S. cerevisiae strain, in which both loci were active, no prioritisation of repair was observed in these loci [1427].

Experiments with agents inducing chromatin hyperacetylation (n-butyrate) in human adenocarcinoma cells showed that n-butyrate facilitated the accessibility to DNA repair enzymes to the chromatin [1428]. Later, it was demonstrated that UV irradiation was associated with histone hyperacetylation at core histones H3 and H4 in yeast, allowing unimpeded access of the DNA repair machinery to the lesion [1429]. Failure to achieve histone H3 hyperacetylation was associated with impairment of repair of UV-induced DNA damage.

Increased repair activity has been detected in the vicinity of matrix attachment sites (MARs) in mammalian and human cells [1430-1433]. Matrix attachment sites (MARs, also known as scaffold attachment regions or SARs) are defined as sequences ≈500 bp long, spaced about every 30 Kb apart from each other and located at sites where the chromatin loops physically attach to the nuclear matrix. MARs are control elements functioning in the maintenance of the chromatin dynamics and the regulation of gene expression. Some MARs may contain origins of replication or enhancers of transcription [reviewed by 1434, see also 1435]. MARs regulate the higher-order organisation of chromatin and the expression of the genes in their vicinity [1436-1438]. Recently, it has been proposed that SARs serve as landmarks in the chromatin architecture, delineating domains with different architecture

and controlling the nuclear DNA transactions in a spatial as well as temporal fashion by facilitating or limiting the access of various regulatory factors to DNA [1439,1440]. It makes sense, therefore, that repair of MARs is carried out with priority, as one a single MAR regulates the function of a much larger genomic region, and unrepaired damage may cause gross disturbances of the chromatin structure. Further hierarchical distribution of repair activities in regions outside the physical limits of the MAR is governed by the priority rules dependent on the transcription state of the genomic region.

Several groups of DNA-binding proteins with known functions in chromatin remodelling have been investigated on order to elicit a relationship with prioritisation of DNA repair in specific chromatin regions. Experimental proof was obtained relatively quickly for high mobility group (HMG) proteins. HMG proteins are chromosomal non-histone proteins with important roles in the maintenance and remodelling of chromatin. They are abundantly and ubiquitously expressed in undifferentiated cells, but are usually present at low levels in somatic cells of adult organisms [1441,1442]. HMGA proteins are master regulators of gene expression, functioning by means of altering DNA conformation [603,605]. HMGB proteins have been shown to play a role in nucleosomal organisation of chromatin and regulation of the expression of various proteins at transcription level, having earned HMGB the name of '*a master DNA repair mechanic*' [1443].

Two alternative models for the function of HMG in DNA repair in living cells were initially formulated. One of these models proposed that HMG proteins actually interfered with repair by binding and physical shielding of damage sites from the repair machinery [1444,1445]. The other model postulated that binding of HMG to damage sites actually served to recruit other repair factors to the damage site and induce repair complex assembly. The first model was supported by the findings that upregulation of HMGA expression was a common finding in virtually all human cancers 606, reviewed in 607 and 1446]. It has been found that overexpression of HMGA may directly inhibit DNA excision repair of any type (nucleotide excision or base excision repair), in the nucleus as well as in mitochondria [608,609]. HMGA may inhibit the expression of protein factors directly involved in DNA repair, such as XPA [1447]. Approximately at the same time, however, it was demonstrated that HMG proteins could modulate the functioning of the cell repair machinery, selectively stimulating or suppressing DNA repair. Experimental proofs have been obtained for the role of HMGB in base excision repair, nucleotide excision repair and mismatch repair. HMGN proteins have been shown to stimulate repair of cyclobutane pyrimidine dimers in vivo, whereas HMGA proteins inhibited NER of CPDs in A-T rich stretches in DNA both in vitro and in vivo. HMGB proteins were found to inhibit NER of cisplatin-induced DNA intrastrand crosslinks, but could either promote the removal of nucleoside analogues from DNA or trigger apoptosis of the cells carrying modified DNA [reviewed in 1448 and 1449]. HMGB1 functions in the recognition of the damage in mismatch repair and regulates sub-pathways of base excision repair (long-patch vs. short-patch) via modulation of the activities of enzymes of BER (e.g. stimulating the flap cleavage activity of FEN1; the incision activity of APE1, and suppressing the 5'-sugar phosphate lyase activity of DNA polymerase β) [1230]. Some HMG proteins have been found to bind with high affinity to sites of abnormal topology in DNA. For example, HMGB1 has been found to

bind to a variety of non-canonical structure –triple-helix DNA, looped DNA and Z-DNA [1450,1451]. Later, HMGB has been found to facilitate DNA repair in regions with triple-helix DNA by binding to the damage site and signalling for recruitment of the NER repair machinery [1443,1452,1453]. Eventually, both models for the regulatory role of HMG in DNA repair were accepted as valid, depending on the type of HMG protein, the type of damage, and the mechanism of repair. It is currently believed that constitutive expression of tumour-specific HMG variants leads to induction of chromatin hyperplasticity in the transformed cells, eventually resulting in ectopic expression of proteins characteristic of the undifferentiated state [607,617,1446].

Lately, it has been hypothesised that the relationship between chromatin structure and DNA repair works both ways – that is, not only the structure of the chromatin in different genomic regions was involved in the distribution of repair activities in the eukaryotic genome, but also the presence of damage in DNA could recruit the cell repair machinery merely by altering the local DNA topology. The latter may, in turn, induce changes in the higher-order structures at the damage site. Indeed, as NER is capable of repairing many different types of DNA damage, there would be no need for precise elaboration of the particular type of damage in order to recruit the repair machinery that repairs it all (except in the cases when it is a double-strand break or a mismatch which may require specific repair mechanisms). ATM has been found to be strongly implicated in relaying the signal for presence of damage in DNA in the non-transcribed regions of the genome, eventually resulting in local relaxation of the chromatin structure so as to ensure easier access of the repair machinery to the damage site [1454,1455]. Thus, the presence of 'open' chromatin conformation at the damage site would make the access of the repair machinery to the damage site easier, and, at the same time, would generate a signal that is amplified and relayed to other signalling and effector molecules. It has already been shown that prolonged binding of DNA repair factors to chromatin (effectively maintaining its 'open' conformation) may activate the DNA damage response pathway even in the absence of real DNA damage [1456].

9. DNA repair and evolution

9.1. Mechanisms of genome evolution

I'm truly sorry man's dominion
Has broken Nature's social union...
Robert Burns, To a Mouse, on Turning Her Up
in Her Nest with the Plough (1785).

Almost 9 million different species of living organisms currently exist on Earth [1457]. There is enormous variety with regard to their morphology, anatomic organisation, physiology and metabolism. All these organisms use nucleic acids to record, store and create copies of genetic information. With the exception of RNA-based viruses and phages, the nucleic acid of choice for storage and transmission of genetic information from generation to generation is usually DNA, with RNA usually used as an intermediate and/or auxiliary medium. The differences between genomes of different species (with regard to size, gene content, etc.)

usually become greater with the species being farther away on the phylogenetic tree (Fig. 26).

There is only several (between 1 and 5) per cent difference between the sequences of human DNA and DNA of great apes [1458,1459]. There is 70–90% similarity between the genomes of humans and mice, with the degree of similarity varying strongly in different regions of the genome. It is very rare, however, not to find a human homologue to any gene in the mouse genome [1460]. The degree of similarity between the human genome (adult organism made of $\approx 10^{14}$ cells) and C. elegans (the microscopic nematode made of $\approx 10^{3}$ cells) is about 50% [1461].

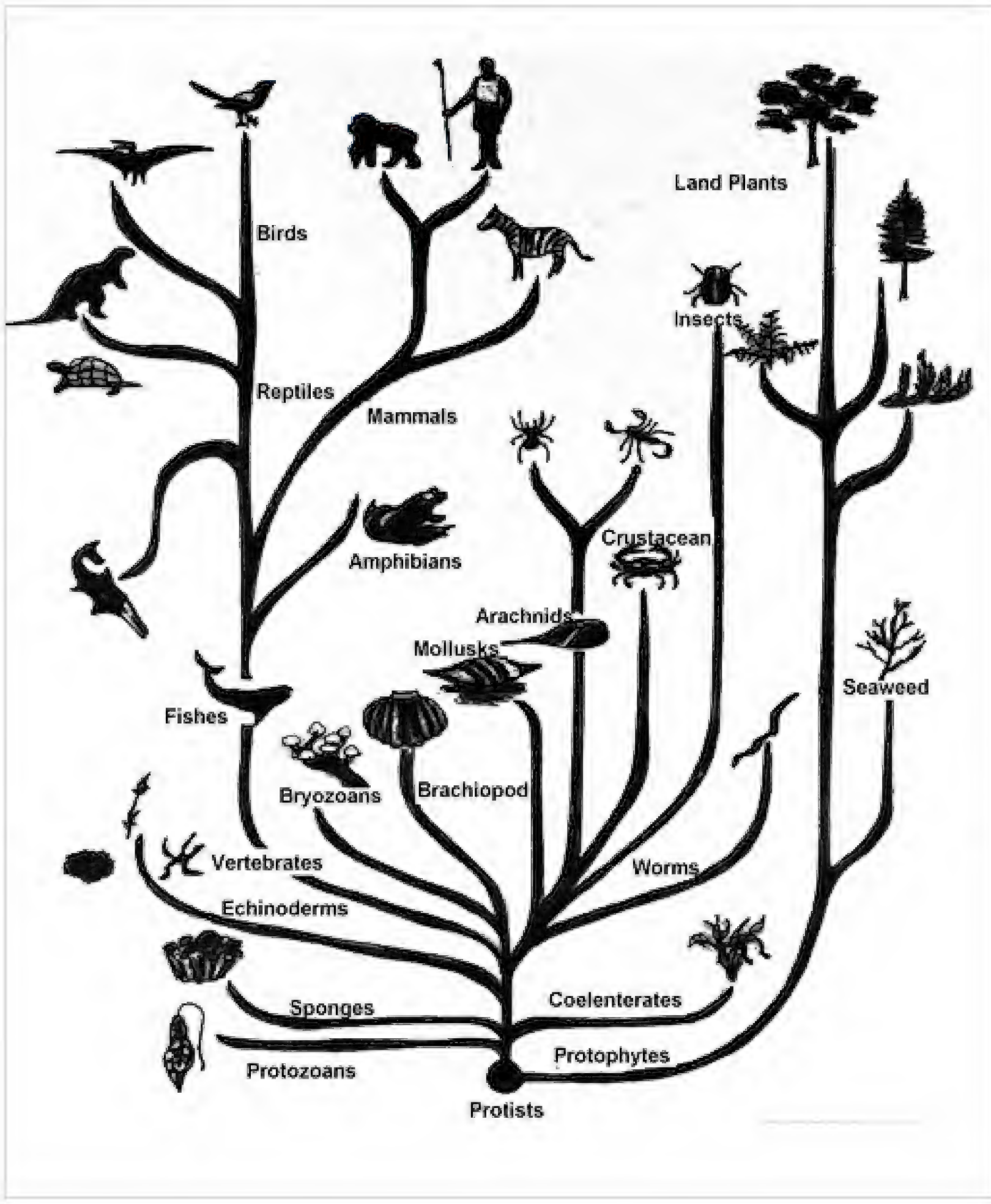

Figure 26. A simplified phylogenetic tree, presenting the course of evolution on Earth. Adapted from "A Simplified Family Tree of Life" in The Evidence of Evolution by Nicholas Hotton III, the Smithsonian, 1968.

Genome size, gene content and DNA sequence may vary between different species. For example, Saccharomyces cerevisiae has a haploid genome of 1.2×10^{7} bp, containing about

7000 genes; Neurospora crassa, also a species of yeast – about $4x10^7$ bp, 10,000 genes; the nematode C. elegans – about $1x10^8$ bp, 21,000 genes; A. thaliana, a flowering plant with a very small genome for a plant– about $1.25x10^8$ bp,27,000 genes; Drosophila melanogaster – also $1.25x10^8$ bp, but 17,000 genes. Mammalian genomes generally contain about $3–7x10^9$ base pairs and 25,000–30,000 genes. It is easy to see that the genome size and the number of genes does not always correlate with the complexity of the organism. For example, Drosophila seems to have about 20% less genes than C. elegans, although insects occupy a higher position in the phylogenetic tree than nematodes. Human genome is twice the size of the genome of A. thaliana, but the gene content is only 11% higher. The genomes of some amphibian species and monocotyledon plants may be very large, even larger than the human genome – about $10^{10}–10^{11}$ bp per haploid genome. The bulk of this DNA, however, seems to be made of non-coding sequences, as the number of genes does not seem to vary significantly compared to the number of genes in other plant species with smaller genomes. In the course of evolution new genes usually appear by gene duplication and subsequent divergence of the copies (gene paralogues). Paralogues are, for example, members of large gene families (e.g. major histocompatibility complex (MHC) proteins, immunoglobulins, G-proteins, homeobox proteins, etc.). The gene copies may remain in the vicinity of the original gene locus or may be moved around the genome by translocation. After they have become separated, the copies usually follow their own course of evolution. Some of the gene copies may eventually be inactivated, but are still retained in the genome as pseudogenes.

It is currently believed that the thousands of different genes in living beings of today have their origin in a small number of ancestral genes that have been altered, duplicated and rearranged ever since the first nucleic acid-based living forms appeared on Earth – that is, over 3 billions of years ago. The homology between different genes, the encoded proteins, or between discrete regions in the macromolecules may be obvious. For example, A-domains of human clotting Factor VIII are homologous to the A-domains of clotting Factor V and the A-domains of both proteins are similar to ceruloplasmin, a protein with ferroxidase activity [1462]. The C-(discoidin-like) domains of Factor VIII are homologous to the C-domains of Factor V, and both are partly homologous to the C-end of discoidin, a cell adhesion protein of the slime mould Dictyostelium discoideum [1463,1464]. Exon-intron boundaries in human genes of Factor V and VIII are 85% identical. Similarly, human clotting proteins Factor VII, IX, X and protein C all contain two epidermal growth factor-like domains, responsible for the binding for cell membranes [1465].

The equivalents of the same essential gene between different species are called orthologues. The sequence similarity of orthologous genes may greatly vary. Some genes are highly conserved and their sequences may differ only slightly in between of different species. Conserved genes are, for example, the genes coding for major factors of the blood clotting cascade, basic transcription factors, receptors, hormones, etc.

As a rule, the coding sequences in a genome are more conserved than the non-coding DNA. This makes perfect sense, as mutations occurring in the coding regions are likely to affect the function of the gene product, while mutations in non-coding regions are usually neutral. There may be exceptions to the latter, for example when mutations in the non-coding

region affect the level of expression of the gene and/or splicing of the transcript. Non-synonymous substitutions of nucleotides and amino acid residues typically occur at a slower rate than synonymous ones, as the risk for producing a phenotype conferring low fitness to its carriers is higher with non-synonymous substitutions.

Highly conserved human and animal orthologues may only differ in one or several nucleotides, which may mean no difference in protein sequence or difference in only one or several amino acid residues. For example, porcine insulin and porcine factor VIII are so similar to their human orthologues that they have been (and sometimes still are) successfully used in replacement therapy for human diseases (insulin-dependent diabetes and haemophilia A, respectively). Human leptin gene differs from chimpanzee leptin gene by only 5 nucleotides (1.1%) (Fig. 27). Four out of these five nucleotide substitutions result in synonymous amino acid substitutions, therefore, on protein level, the difference between human and chimpanzee leptin is in only one amino acid residue (0.7%), a valine-to-methionine substitution (Fig. 27). Human recombinant leptin has been shown to be capable of cross-correcting infertility in obese leptin-deficient mice [1466].

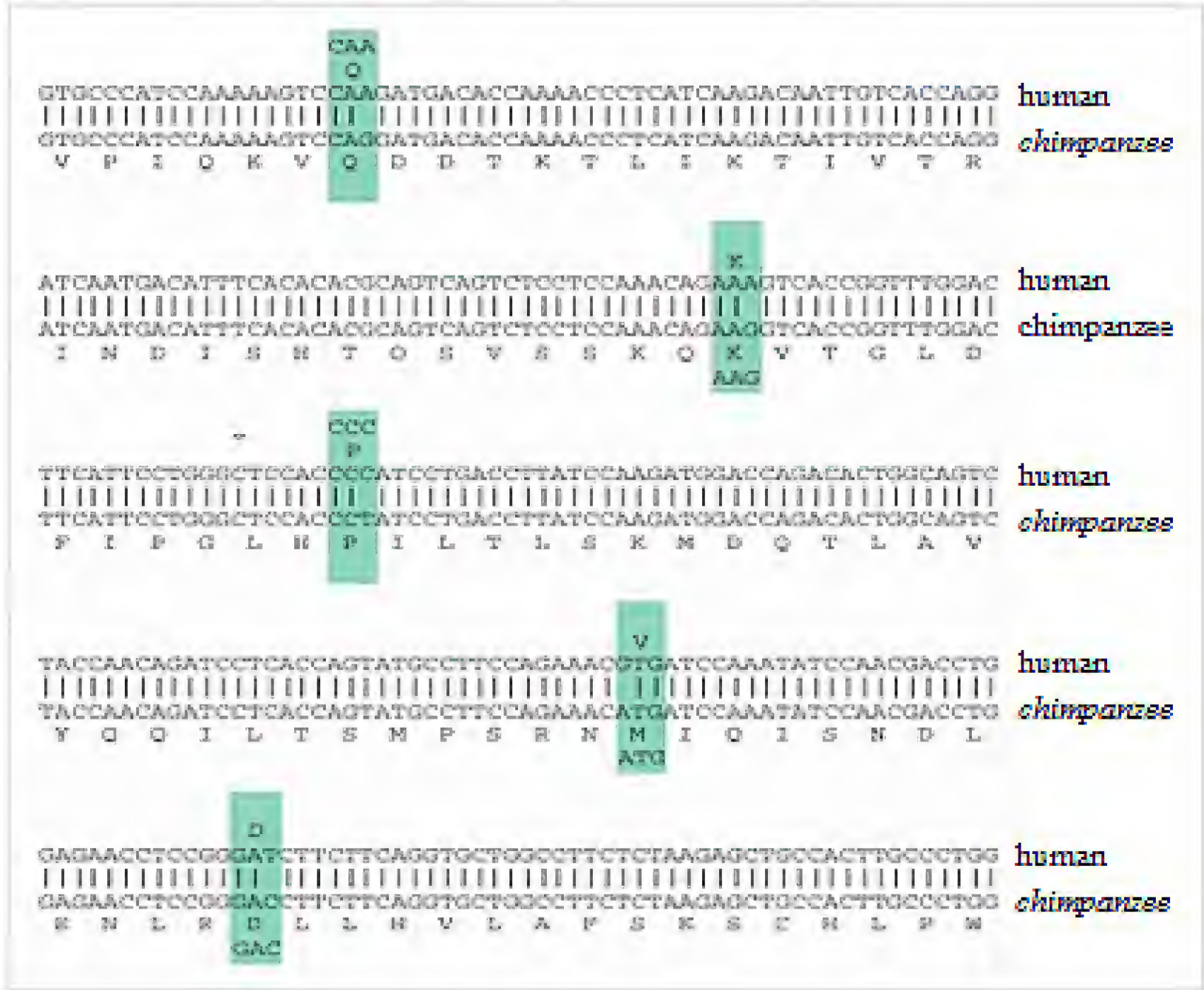

Figure 27. Variance in nucleotide (above) and amino acid (below) sequence of human and chimpanzee leptin. Only the first 300 nucleotides of leptin gene sequence are presented on the figure, as the remaining 141 bp sequence from the 3'-end is identical between the two species.

Quite often, conserved sequences are virtually identical between different species, but may be placed in different locations within the genome. If we use the example of man and the mouse again, the similarity between their genomes may vary from 70 to 90% in different regions, but the percentage of synteny (the similarity in the linear arrangement of genomic

sequences along the length of chromosomes) is very high, over 90%. The X chromosomes in both species are made of practically identical syntenic blocks. Other genomic segments may be similar in sequence and gene content but are located on different chromosomes, as a syntenic block or in smaller segments dispersed over a larger genomic sequence. For example, an orthologous sequence forming a syntenic block on the mouse chromosome 11 is located on the human chromosome 17, with the block broken down in 16 smaller segments separated by intervening DNA sequence. The divergence of rodents as a separate branch in mammalian evolution is dated to have occurred about 60 million of years ago. It is believed that about 300 genomic rearrangements account for the differences in the syntenic maps of man and mouse [1467]. This means that a major rearrangement had occurred, on the average, every 200,000 years.

The time of divergence between the primitive Old World monkeys and the ancestral hominids is believed to had occurred about 30 million years ago [1468]. About 1 500 inversions of genomic fragments have occurred in the period of divergence between the man and the chimpanzee. The sequences of the inverted fragments are usually very similar between the two species, between 97 and 99% [1469]. For example, a specific 9.5 Kb repeated unit in intron 22 of the gene coding for the blood clotting Factor VIII (F8) is very similar in sequence between the different members of the Hominidae family – orangutans, gorillas, chimpanzees and humans, but the number of the copies of the sequence and their orientation in the genome are different in the different species. In humans, the 9.5 Kb fragment is repeated at least two times on the same X chromosome, but outside the Factor VIII gene, at distances of several hundred Kb from the F8 gene locus (Xq28). Sometimes, albeit rarely, there may be three or more repeated units of the 9.5 Kb region of intron 22 per human X chromosome, with very high degree of homology between them (over 99.9%) [1470]. Between humans and other primates, the copies of the 9.5 Kb repeated region in intron 22 of the F8 gene exhibit ≥99% similarity.

Gene copies that have arisen from duplication of an ancestral gene and subsequent independent evolution of the resulting copies usually retain regions of high homology, however, divergent from one another they might have become. Translocations, duplications and deletions (resulting from recombination between direct copies of the same sequence or between regions of partial similarity between different sequences) and inversions (resulting from recombination between inverted copies of sequences with high degrees of similarity) add to the genetic diversity of eukaryotic genomes. Balanced genomic rearrangements, even large ones, may be asymptomatic and may only be discovered when an unbalanced translocation becomes manifest in the offspring of parent/s that are carriers of balanced rearrangements. Many genes can actually perform normally with a single genomic copy only, provided that this copy is not subject to epigenetic modifications (though there is risk for somatic inactivation of the remaining gene copy). For example, the hybrid genes resulting from recombinations between regions of homology in delta and beta genes in the human beta-globin gene cluster may be transmitted by asymptomatic heterozygous carriers through many generations. When these defects are co-inherited in compound heterozygous state with other deleterious mutations in the beta-globin gene, the resultant phenotype may be intermediate or severe thalassemia [1471-1473]. The

human beta-globin gene cluster contains 5 active genes located on 11p chromosome arm, in the 5'ε-gamma G-gamma A-δ-β-3' order. The arrangement of the genes in the cluster generally reflects the sequence of their expression during individual development, although different lineages of haematopoietic progenitors may show slight differences in the timing of expression of different globin proteins [1474] Recombination between the delta and beta genes results in fusion genes, producing the fusion globin variants Hb Lepore (delta-beta) and Hb anti-Lepore (beta-delta) [1475-1476].

9.2. Sources of genetic diversity in evolution

Add the ingredients while mixing,

first on low speed, then increase speed.

A standard instruction in cooking

There are three major sources of genetic diversity in species with sexual reproduction – spontaneous mutagenesis (including error-prone template copying and end joining) and genetic recombination in meiosis All of these are essentially stochastic processes, that is, there is virtually no way of predicting the exact location where alterations of the DNA sequence might occur. Indeed, there are regions of the genome where the recombination rate is high (recombination hotspots, an estimated 25,000 in the human genome) and regions with very low frequency of rearrangements during meiosis (e.g. the MHC cluster at 6p21.3; the Y-chromosome outside the pseudoautosomal regions, and others) [1477]. Nevertheless, recombination may occur virtually anywhere in the genome, provided that it is not too close to another site of recombination. Spontaneous mutagenesis is also random, although there are mutation hotspots as well. As the coding sequences in mammalian genomes constitute only several per cent of all the DNA, the likelihood that a genomic alteration would occur in the non-coding DNA than in coding DNA is high. There is, however, considerable difference in the heritability potential of DNA alterations occurring in coding and non-coding regions. Genomic alterations in coding DNA transmitted down the vertical line (parents to offspring) may be associated with phenotypes unfit for independent life or rendering the carriers incapable of reproduction. Thus, allelic variants that put the carriers at a disadvantage may be rapidly removed from the genetic pool. Neutral or beneficial (much less frequently occurring) mutations in the coding regions of the genome and alterations in the coding regions associated with a potential disadvantage manifesting in later life (when the carrier individual had already transmitted the mutation to their offspring) may also be retained in the genetic pool. Mutations occurring in the non-coding regions of the genome are usually associated with no change or only a subtle change in the phenotype and are, therefore, readily retained in the genetic pool. Thus, there is a selective pressure on the sequence in the coding regions of the genome to remain as they were (or as close to the original sequence as they could possibly be) during the course of evolution, whereas the genetic variety in the non-coding regions may be higher. Differences in the DNA sequence of non-coding regions may exist even between individuals of the same species. The latter forms the basis of genetic analysis for individual identification and population and species affiliation.

Some of the genetic variance of genomes is due to the activity of mobile genetic elements. Mobile genetic elements may be of exogenous origin (e.g. retroviruses) or endogenous

(plasmids and group II introns in bacteria and plant organelles; elements with transposon-like activity that are remnants of ancient retroviral activity; reverse transcripts of RNA that are capable of incorporation into DNA genomes, etc.). Mobile genetic elements may move around the genome using a cut-and-paste mechanism (maintaining roughly the same number of elements per genome – e.g. Class I transposons) or copy-and-paste (increasing the number of elements per genome – Class II transposons (retrotransposons)). They may translocate elements of the genome to various ectopic sites, causing alteration, loss or gain of various sequences and activation and/or inactivation of various genes [1478-1480]. Thus, mobile genetic elements may also produce potentially heritable allele variations and emergence of new alleles.

The survival of a species for long time (at least hundred thousand years or more) is usually a question of successful adaptation to changes in the environment. Capacity to adapt is directly linked to genome plasticity, that is, mutability and capacity to tolerate mutation burden. Nature, however, has not equipped living cells with specific mechanisms for targeted introduction of genome alterations, apart from the specialised low-fidelity DNA polymerases, the error-prone mechanism of NHEJ and meiotic recombination. The former are employed rarely, usually as an emergency mechanism for short-term survival of cells with damaged DNA. Recombination is a routine mechanism for reshuffling pre-existing allelic variants to create new combinations, but as a mechanism for creation of genomic diversity, recombination is typically used in gametogenesis only. Currently, there is no known in vivo mechanism for premeditated introduction of particular mutations into specific genomic regions. The mechanisms for creating genomic diversity (and, therefore, adaptivity) operate at random and normally their effects only begin to show after a long time. In the eukaryotic genome, the rate of occurrence of fixed mutations in coding DNA producing exchange of one codon to another may vary, depending on the average gene size in the genome (the longer the gene, the higher risk of introduction of alterations); the cycling rate of cells, and the resources for repair of DNA damage. For example, in the mitochondrial DNA, where repair by NER is specifically inhibited and meiotic recombination is hardly impossible, the rate of occurrence of mutations is faster than in the coding portions of the nuclear DNA [1481]. The rate of introduction of mutations may vary for different organisms, but it had been estimated that several nucleotide changes (1–5) per 10^9 nucleotides occurs per replication cycle. In a protein about 400 amino acids long, coded by a nuclear gene, a heritable amino acid change would occur in the germline roughly once every 200,000 years [1482]. It has been calculated that in a small population of eukaryotes (about 10,000 individuals) with size of haploid genome 10^8–10^9 bp and mutation rate about $5x10^{-9}$ bp, every possible nucleotide substitution would occur several dozens of times within 1 million years. The evolution of life on Earth has been going on for more 3 billion years now, therefore, there has been plenty of time in which to test and re-test new genetic combinations. Many of the potentially viable genetic combinations had probably died out during any of the large extinction events in the past, therefore, adaptability does not guarantee that a genetic alteration, even a neutral or a beneficial one, would be retained and passed on.

If each and every genetic alteration was repaired with 100% fidelity, and/or all cells carrying genetic alterations were always eliminated from the population, no new genetic variants would ever arise. In evolutionary terms, resistance to change is not a good option. This may be best illustrated using an example of currently existing natural populations that had experienced at some time in the course of their evolution a population 'bottleneck'. 'Bottleneck' is a term used to describe a topological site and/or point in time where the performance and/or the capacity of a complex system may be restricted by a limited number of components or resources. A population 'bottleneck' specifically means that at some point the number of individuals in a population had become severely limited. Even in normal-sized populations only a small percentage of the individuals produce offspring (and, respectively, only their genes are transmitted to the next generation), but in 'bottlenecked' populations, the genetic pool may consist barely of several dozens or hundreds of individuals. In a population made of small number of members, the diversity of the genetic pool progressively decreases and the members of the population may become genetically very similar to one another. Such is the case, for example, with the natural populations of the cheetah (Acinonyx jubatus). The 'bottleneck' in cheetahs had occurred relatively recently, during the last glacial period (10–12,000 years ago). It is believed that only one species survived from the several previously existing species of cheetah and the number was severely reduced. These surviving cheetahs became the ancestors of all currently living cheetahs, out of a genetic pool made of several hundreds or thousands of genomes only. Cheetahs of today suffer badly from the consequences of the limited genetic diversity resulting from this 'bottleneck'. The population trend of the species is currently decreasing. The fertility in cheetahs, free-living as well as in captivity, is very low, and only 5–10% of cubs in every litter survive. There is a high rate of multiple paternity in cheetah litters [1483]. The promiscuity observed among female cheetahs is believed to have developed after the 'bottleneck' had occurred, as a compensatory mechanism to counter the excessive litter mortality [1484]. A. jubatus is currently classed as a vulnerable species (threatened by extinction in the wild) in the Red List of the International Union for Conservation of Nature (IUCN, for details see IUCN red list A. jubatus). MHC antigen diversity in cheetahs is very low and skin transplants between unrelated individuals are very rarely rejected [1485,1486].

Somewhat similar is the situation with the currently existing populations of the Northern elephant seal (Mirounga angustirostris). Man is directly responsible for the dwindling of the populations of the elephant seal down to several dozens of individuals in XIX century. After measures for preservation of the species were put in place, however, the size of the populations of the Northern elephant seals has considerably grown. The population is currently estimated at ≈170,000 members and growing in size. From once being practically extinct, the species has been reclassed as 'least concern' in the Red List of IUCN (IUCN Red List M. angustirostris). The levels of genetic diversity in the Northern elephant seal populations is very low, but the populations are not in decline, quite the contrary [1487,1488].

The extinction of ancient species of cheetahs is believed to have been a result of environmental changes and/or a catastrophic event. Only small group/s of related animals survived, and it is likely that misadaptation was the reason for all but one species to die out.

Northern elephant seals were brought to extinction by man, and it is highly unlikely that preference played much of a role in the sealing expeditions – the hunters simply killed all seals they could get, regardless of their genetic background. Therefore, the populations of seals that survived the hunts of XIX century were small indeed, but it is likely that the internal genetic diversity was not severely affected. This may explain at least partially why those two bottlenecked populations currently exhibit very different population trends.

9.3. Evolution of DNA repair

Don't ask me how this mechanism actually works... It works, however, so that in a billion of years those works of yours, combined with the works of millions of others, would not bring the end of the world. Not the end of the world in general, of course, but the end of that world ... that existed a billion of years ago, the world whose existence you all unsuspectingly threatened with your microscopic efforts to bring order out of the chaos.

Arkadiy and Boris Strugatskie, One Billion Years
Before The End Of The World (Definitely Maybe), 1974.

9.3.1. We were all created (kind of) equal

If we compare the mechanisms of DNA repair between different groups of organisms that are placed very far apart from each other on the evolutionary tree, it is obvious that they would be virtually identical or, at least, very similar. All creatures currently living on Earth possess a diverse set of mechanisms for repair of DNA – namely, excision repair (BER and NER), mismatch repair, repair of breaks in DNA, and, in some organisms, mechanisms for direct repair of DNA (e.g. photoreactivation). The specific 'tools', however, may be different for the different groups. For example, complex eukaryotic organisms have lost their capacity for direct repair of DNA by photoreactivation, albeit they have retained the flavin core of the photolyase as a component of the machinery for regulation of circadian rhythms. Eukaryotes, however, possess a complex mechanism for repair of double-strand breaks by non-homologous end joining while bacteria only possess the minimal set of NHEJ proteins. Until about a decade ago, NHEJ was believed not to exist in bacteria at all, but in 2002, a protein complex capable of joining free DNA ends sharing only minimal homology was identified in bacteria [1489].

Cell organelles specialised in energy conversion such as mitochondria may only maintain the mechanisms for repair of the most common type of damage to their DNA (oxidised nucleotides and double-strand breaks). Thus, the nucleotide excision repair is not used in mitochondria and plastids [21,1490]. The basic repair mechanisms employed in mitochondria and plastids are BER, mechanisms for repair of double-strand breaks and mismatch repair. The mechanisms of repair and the signalling and effector molecules for repair of mitochondrial DNA are the same as in repair of nuclear DNA. None of the proteins acting in repair of organelle DNA is coded by their own DNA. Instead, the nuclear genes in are transcribed, the respective proteins are synthesised in the cytoplasm and are subsequently imported into the organelle. Mitochondria and plastids are currently believed to have arisen by endocytosis of ancient prokaryotes capable of energy conversion by oxidation (purple non-sulphur bacteria for mitochondria and cyanobacteria for plastids, respectively) by primitive eukaryotic cells, which subsequently developed into symbiosis [reviewed in 1491 and 1492]. This is believed to have occurred about two billion years ago

for mitochondria and one billion years ago for chloroplasts [1493-1496]. Alternatively, repair by nucleotide excision might have initially existed in organelles but might have been lost in the course of evolution because it turned out to be redundant.

The shared features of DNA repair in different organisms have laid the basis for the current concept that the mechanisms of DNA repair had been established early in the course of evolution. The differences between molecules and mechanisms of repair in different groups of organisms supposedly emerged later (sometimes, much later), as a result of independent evolution. In other words, from the very beginning of life on Earth, the basic mechanisms to protect the main information carrier molecule from damage were in place, and the evolution had only had them slightly modified in order to suit the needs of the various organisms. Examples in support of this theory are numerous. In Archaea (considered to be phylogenetically very old) were found proteins strongly resembling in major aspects of structure and function some of the proteins of nucleotide excision repair. Among these were proteins similar to the helicases XPB and XPD, the endonuclease XPF and the auxiliary protein PCNA [1497-1499]. It is notable that the repair proteins of Archaea exhibit more similarity to the homologous proteins of currently living eukaryotic cells than to prokaryotes [1500]. This and other findings were among the proofs in support of the concept that Archaea do not belong with the kingdom of Bacteria, but are a separate branch of evolution. The same peculiar similarity between Archaea and eukaryotes rather than prokaryotes was found for proteins acting in transcription and DNA replication [1501].

Some of the proteins of DNA repair may have developed later in evolution. For example, the XPA protein is present, with small variations, in all eukaryotes, but in bacteria the major recognition molecule – UvrA – bears no similarity to eukaryotic XPA [1502]. Among eukaryotes, however, the similarity of orthologous *XPA* gene sequences is very high, even among very distantly related species. For example, up to 52% identity and 70% similarity at amino acid level were observed between XPA proteins from Hydra spp. and from various other animals, including various mammals (mice, dogs, cattle, chimpanzees, and man) [1503]. In some regions of the compared proteins, the identity reached 71% and the similarity 91% between Hydra and man.

There is no known homologue of XPC in prokaryotes either. This makes sense, as virtually all DNA in prokaryotes is transcribed; therefore, there is no need for a designated mechanism for detection of damage in transcribed and untranscribed DNA.

The major eukaryotic genes coding for proteins acting in DNA repair are typically conserved between unrelated species and the degree of similarity may be quite high, even between very simple eukaryotes and higher animals. For example, the yeast homologues of XPC are the Rad4 protein (in S. cerevisiae [1504]) and the Rhp41 and Rhp42 proteins (in Schizosaccharomyces pombe [1505]). XPC protein from unrelated species are quite similar to one another, especially in their C-terminal portion –50% identity (68% similarity) between Drosophila XPC protein and the human XPC protein, and 27% identity (48% similarity) between Drosophila XPC protein and the S. cerevisiae Rad4 protein [1506]. The degree of identity between human XPC and yeast Rad4 proteins is 27% and between human and mouse XPC – 73%. Human and yeast XPC orthologues exhibit approximately equal binding affinity to damaged DNA [1507]. The degree of identity at protein level

between human XPD protein and the yeast Rad3 is about 53% (72% similarity) [1508] and between XPD proteins of man and Drosophila is even higher, about 68% (83% similarity). Sometimes, trans-complementation may be possible, that is, genes or gene fragments from one species may be incorporated into the genome of other species, where the heterologous gene would function normally or at least as close to normal as possible. For example, yeast and Drosophila homologues of repair proteins had been shown to cross-complement defects in human cells deficient in components of the repair complex [1509-1511].

Some authors believe that the genes coding for proteins of DNA repair are conserved between different species because they are inherently capable of catalysing more than one type of reaction ('catalytic promiscuity') [1512,1513]. For example, the E. coli protein AlkB catalyses removal of alkylated bases regardless of the type of base (purine or pyrimidine); the location of the modified base within the DNA sequence; and the length of the alkyl residue. Uracil-DNA glycosylases remove not only uracil from DNA, but also 5-hydroxyuracil; alloxan (2,4,5,6-tetraoxypyrimidine), and others. In the case of a 'promiscuous' enzyme, the selective pressure for introduction of genetic changes to suit different needs (e.g. to produce several different molecules capable of catalysing reactions using different substrates) would be weaker. The gene would still change and evolve, but at a considerably slower rate.

There are, however, exceptions to the 'promiscuous enzyme' theory and sometimes molecules of DNA repair may exhibit specific properties even within the same group of organisms, For example, photolyases from different bacterial species may exhibit different substrate specificity – some would work on thymine dimers only, others on 6-4 photoproducts, and some on nucleotides affected by any of the two types of photodamage, although the general mechanism of repair in all the three cases is essentially the same.

Almost all repair genes and proteins are conserved between different species of eukaryotes. There is, however, one notable exception to this – namely, the proteins acting in repair by non-homologous end joining (NHEJ). NHEJ genes and proteins exhibit an unusually accelerated course of evolution compared to other proteins acting in repair of damaged DNA or repair-associated signalling [1514]. As NHEJ is a major mechanism for rearrangement of sequences coding for variable regions associated with antigen recognition in immunocompetent cells, its accelerated rate of evolution is considered by some authors to be associated with the rates of viral evolution. As viruses mutate readily, the immune systems of their prospective hosts must keep pace with it [1514,1515]. Notably, the products of some of the genes coding for proteins acting in repair of double-strand breaks (the MRN (MRE11-RAD50-NBS1 complex) may act as inhibitors of formation of viral particles by induction of concatemerisation of viral genomes in aggregates too large for packaging, and may be a target for inactivation by some viruses [1516,1517]. NHEJ proteins have also been found to bind to retroviral proteins, cDNA, and pre-integration complexes [1516,1518,1519]. The explanations proposed so far have been that NHEJ proteins were actively recruited by the viral complex to protect free viral cDNA ends so as not to alert the cellular machinery for repair and induction of apoptosis of their presence and/or that the host NHEJ proteins were called upon sites of retroviral cDNA integration in order to repair the breaks after the DNA copy of the viral genome had been inserted into the host genome.

9.3.2. How evolution may actually go wrong by doing everything right – the example of the X chromosome

Yet Nature and Nature alone is the procreator of the world... the mad,
all-powerful, all-purblind, accidental and chaotic Nature.
Milen Ruskov, Thrown into Nature (2011).
Translated by Angela Rodel.

When we talk about evolution, it is almost always in the past tense, as of something of very long ago. In fact, the evolution continues in real time, only the rates of emergence of perceptible changes are very low. The effects of evolutionary selection do not normally become apparent within the average human lifespan, except in the rare cases of gross failures of the ongoing testing of genetic variants. These failures usually manifest as sporadic occurrence of genetic disease in a family without history for the disease or related diseases and conditions.

In only a proportion of sporadic cases of genetic disease, the mutation/s responsible for the disease phenotype have been transmitted for generations in latent (e.g. heterozygous) state. Severe genetic disease often occurs because of new (de novo) mutations that have arisen in the recent generations – the grandparent/s, the parent/s, or early in the embryonic development of the affected individuals. Some genetic diseases with dominant mechanism of inheritance occur exclusively de novo, as the affected individuals may not survive childhood and adolescence and/or be incapable of reproduction (for example, patients with Hutchinson-Gilford syndrome rarely live beyond their early teens). Sporadic genetic disease transmitted in autosomal recessive pattern may occur because of compound carriership of an inherited mutation that has been segregating in the family for a long time and a de novo mutation occurring in the parental germline or during the early embryonic development of an individual that is heterozygous for the familial mutation.

The X chromosome in mammals and in man is a major hotspot for de novo mutations. For the majority of genes that exist in two copies per genome in somatic cells, one intact gene copy is often sufficient to ensure normal functioning. De novo mutations on the X chromosome usually manifest as X-linked recessive disease in males, as they carry only one X-chromosome, but may sometimes cause emergence of symptoms of the X-linked disease in female carriers (e.g. because of biased X-inactivation). Some of the relatively rare cases of diseases with X-linked inheritance in women may be attributed to compound heterozygocity for a familial mutation and a de novo mutation [1520].

One of the major causes for the increased rate of de novo mutations on the X chromosome is that the opportunities for pairing with the Y chromosome for the purposes of meiotic crossover during prophase I of male meiosis is very limited. The X chromosome could only pair properly and engage in homologous recombination with the partnering X chromosome during meiosis in females. The X chromosome is quite large, approximately 155 Mb in length [1521] and containing between 1000–2000 functional genes and about 1000 pseudogenes. Less than 30 genes on the X chromosome are shared with the Y chromosome [1522]. During meiosis, X and Y chromosomes only pair within their pseudoautosomal regions (PAR 1 and PAR 2), located in the terminal regions of the chromosome arms. Deletion of parts of PARs or the whole locus together with some of the adjacent sequence is

not uncommon in humans. The genomic fragments containing the missing genes are usually translocated to the other chromosome of the pair (X or Y) chromosomes [1522,1523]. Sex determining genes such as SRY (sex-determining region of Y) are located near the pseudoautosomal boundary and may be translocated together with the PAR region, resulting in sex reversal syndromes – (46, XX) phenotypic males, carrying a fragment of the Y chromosome on the tip of one of the X chromosomes; and (46, XY) phenotypic females carrying an Y chromosome that has lost some of the sex-determining genes on the Y chromosome.

Recombination has been (and currently is) at work at the X chromosome. The X chromosome contains significant amount of repeated sequences. Interspersed repeats account for more than half of the X-chromosomal euchromatin [1524]. Repeated units elsewhere on the X chromosome may mispair during meiosis, becoming potential breakpoints for intrachromosomal recombination. When the pairing repeats are orientated in the same direction to one another (direct repeats), the sequence between them may be lost (deleted) or duplicated. In recombination between repeats that are orientated in the opposite direction, the intervening sequence is usually inverted and may be moved from its original position, sometimes at a considerable distance (see below). This occurs predominantly during male meiosis, and many X-linked diseases that have been traditionally viewed as transmitted exclusively through the maternal line were revealed to be products of mutations occurring in the male germline [1525,1526], for more details see below. Several monogenic diseases (haemophilia A, Duchenne/Becker muscular dystrophy, and others) and even chromosome diseases (some variants of Turner syndrome) have been found to be, in fact, genome rearrangement disorders.

Replication-based mechanisms (break-induced replication, fork stalling and template switching – FoSTeS), etc. are likely to account for significant part of the rearrangements in the human genome, asymptomatic as well as symptomatic. BIR is a mechanism for management of double-strand breaks occurring in DNA molecules that are currently being replicated. It may cause incorporation of DNA sequences from other loci at the breakpoint junctions [1527,1528], increasing the number of copies of these sequences per genome. Carriership of multiple repeated units may not affect the health and/or the reproductive fitness of carrier individuals. Subsequent recombination between the newly generated regions of homology may, however, result in translocations, deletions and duplications (multiplications) [1528]. Fork stalling and template switching (FoSTeS) is another mechanism of homologous repair used for templates that are currently in replication. It uses regions of microhomology between sequences that are within the range of separate replication forks to cause switching of templates between forks. Thus, sequences normally located in different positions in the genome may be joined together, and non-reciprocal rearrangements may be generated [322,323].

Several de novo rearrangements in major genes on the human X chromosome occur with relatively high frequency in all populations. These generally manifest with severe genetic disease in males, transmitted by carrier females that are usually born to non-carrier parents. The most prominent examples are presented below.

Rearrangements in the *F8* and *F9* genes, resulting in sporadic cases of haemophilia A and B

In haemophilia A, recombination between the homologous sequences in the 9.5 Kb inverted regions in intron 22 and outside the gene causes translocation of a large portion of the gene (exons 1–22 and intervening genomic sequence), at a distance of 300–400 Kb from exons 23–26. The translocated fragment is orientated in the opposite direction to the direction of transcription of the *F8* gene [1529,1530]. The 9.5 Kb repeated unit of intron 22 contains an internal CpG island that serves as an origin of transcription for two transcripts: one transcribed in a direction opposite to the direction of *F8* gene transcription (*F8A*) and the other transcribed in the same direction as the gene for Factor VIII (*F8B*) [1470]. Thus, as both portions of the broken gene possess their own CpG islands, the transcription of the two sequences (exon 1–22 and exon 23–26) is unimpeded, but full-length transcript of Factor VIII gene cannot be assembled, resulting in severe deficiency of Factor VIII protein [1529,1530]. The intragenic repeated unit may recombine with any of the extragenic copies, but the distal copy (inversion type I) is used in recombination more often than the proximal copy (inversion type II) [1531]. If there are more than two extragenic copies, any of these may serve as a breakpoint in inversion (type III), with preference given to copies located farther away from the *F8* gene locus. The recombination occurs mainly during meiosis in unaffected males, although it may occur during female meiosis as well. Thus, the somatic cells of these males carry intact X-chromosome, but some of their germline cells may carry X chromosome with inversion in the *F8* locus (germline mosaics) [1532]. These males usually have healthy sons (as the X chromosome is supplied by the mother) and may have daughters that are carriers of the inversion and are, therefore, at 50% risk for having sons affected by haemophilia A. The mispairing of homologous sequences and the subsequent recombination occurs at random. Therefore, the incidence of haemophilia A due to inversion is roughly equal in all populations, about 1:20,000 males [1533, 1534]. The reported incidence of haemophilia A is estimated to be about 1:8,000–1:9,000 live-born boys. It is believed, however, that these estimates are biased towards the more severe forms, as the milder forms may be underreported. Thus, the incidence may be closer to 1: 5000. The inversion with breakpoints in the 9.5 Kb repeated units of intron 22 accounts for about 50% of the severe cases, which are about half of the all the cases, therefore, the incidence of newborn males carrying inversion is about 1:20,000. There might be variations between the incidence of de novo occurrence of type III inversions, as the carriership of more than two extragenic copies of the 9.5 Kb repeated region is not associated with any discernible effects on the phenotype, and, therefore, founder effects may play a role.

It is believed that the ancestral sequence of the repeated 9.5 Kb extragenic unit became duplicated at least 25 million years ago, as highly homologous sequences had been found in monkeys (chimpanzee, African green monkey and Rhesus monkey, diverged from the common ancestor about 30 million years ago). The intragenic copies showed divergence under 1% between humans, chimpanzees, African Green monkeys and Rhesus monkeys, respectively. The repeated units and the flanking sequences were estimated by Bagnall et al. to have evolved at a rate of about 0.1% per 1 million years during the divergence between humans and primates, but significantly slower in the line of divergence between chimpanzees and humans [1535,1536]. Inversions affecting repeated sequences from intron 22 of canine Factor VIII gene and an extragenic copy may be seen in dogs (diverged from the

ancestors of humans ≈94 million years ago) [1537]. Similar inversions have not, however, been described in mice that also have only one extragenic copy of the repeated sequence [1538]. The last common ancestor of mice and humans of today lived about 92 million of years ago. The exon 1–22 inversion in the Factor VIII gene has not been described in other domesticated animals such as sheep and cattle as well [1539-1541]. The latter are believed to have diverged from the common ancestor about the same time as the dogs, about 94 million of years ago [1542]. The occurrence of the third extragenic copy must have occurred relatively recently (in evolutionary terms), as the chimpanzee and the gorilla have three copies of the 9.5 Kb repeated region per X chromosome, as humans usually do, whereas the orangutan and the pygmy chimpanzee that are more distantly related to humans have only two copies of the 9.5 Kb repeated unit [1543].

Partial or complete *F8* gene deletions are found in about 5% of patients with severe haemophilia A. Some of these are due to recombinations with breakpoints in interspersed repeated sequences (Alu, LINE1) [321,1544]. Alu elements are members of the SINEs (short interspersed repeats) family, a repeated sequence ≈300 bp long that is usually seen in multiple copies (≈$3x10^5$) in the haploid human genome. Alu elements exhibit significant sequence similarity to the 7SL RNA (signal recognition particle, SRP, one of the small cytoplasmic RNAs), and are believed to be processed pseudogenes of 7SL RNA [1545]. LINEs (long interspersed elements) are repeated sequences about 7000 bp long, containing two open reading frames. The most common LINE (LINE1) is observed in about 2–$4x10^3$ copies per haploid human genome. SINEs and LINEs may move around the genome, usually by a 'copy and paste' mechanism and are classed as retrotransposons (class II transposons). The index patient carrying the *F8* gene deletion is usually the first case in the family, born to a carrier mother [1546,1547].

Homologous recombination between repeats producing deletions or inversions in the Factor IX (*F9*) gene have been found to be the most common mutation causing Haemophilia B as well. The male/female ratio of occurrence of mutations in the *F9* gene has been found to be about 4 [1548].

Rearrangements in the dystrophin gene, causing Duchenne/Becker muscular dystrophy

Another commonly used example of ongoing molecular evolution is DMD/BMD muscular dystrophy. It is caused by mutations (most commonly, deletions) in the gene coding for the sarcolemmal protein dystrophin (Xq21). The dystrophin gene is one of the largest genes in the human genome (79 exons and 2 300 kb of genomic DNA, which make up for about 1.5% of all X-chromosome). It takes about 16 hours for the gene to be transcribed and the splicing begins before the transcript is even complete [1549]. Genomic rearrangements (mainly deletions, but also duplications and inversions) are responsible for over 60% of the cases of DMD/BMD [1550]. The sites of rearrangements in the dystrophin gene often contain short regions of homology with other sites on the X chromosome that may mediate intra-chromosomal pairing during meiosis – specifically, male meiosis [1551,1552]. In 2009, a recombination-related mechanism was proposed to explain the apparent genomic instability in the two major mutation hotspots in the dystrophin gene – the regions between exons 8 and 13 and exons 45–52 [1553,1554]. In another, more recent study, comprised of DMD/BMD cases as well as patients with other X-linked disorders, it was

found that regions of microhomology (2–10 bp) were observed at breakpoint junctions in about 60% of the study cases. According to the authors, these were most likely remnants of template slippage during recombination by the break-induced replication mechanism [1555].

Turner syndrome with other than 45 (XO) karyotype

Turner syndrome results from complete or partial monosomy for the X chromosome. The typical (45, XO) karyotype is seen in about half of the patients with Turner syndrome. The second most frequent karyotype in Turner syndrome, however, ≈20% is characterised by a isodicentric X chromosome made of two q-arms, so that the cell is partially monosomic for the p-arm of the X chromosome [1556]. The breakpoint regions in these isodicentric X chromosomes often contain inverted repeats. It is believed that most of these Turner syndrome variants result from non-allelic homologous recombination between palindromic sequences [1557,1558]. Non-allelic homologous recombination (NAHR) occurs between DNA sequences which exist in more than one copy and exhibit high sequence homology, but are not polymorphic forms of the same allele (e.g. example, functional gene copies and pseudogenes, copies of repeated genetic elements of the LINE type)

Other genetic diseases due to recurrent rearrangements in the human genome

Among these are X-linked diseases (Pelizaeus-Merzbacher disease; mucopolysaccharidosis II, Hunter syndrome; some of the diseases and conditions characterised by neurodevelopmental delay, and others), as well as diseases associated with mutations in autosomal genes (e.g. spinal muscular atrophy; Charcot-Marie-Tooth disease type IA, and others).

Pelizaeus-Merzbacher disease (PMD) is a X-linked hypomyelinating adrenoleukodystrophy (Xq22) which may be caused by duplication of parts or the whole gene coding for one of the components of the myelin sheath of axons of the neurons in the central nervous system – proteolipid protein 1 (*PLP1*) [1559]. The duplications occur predominantly in the male germline [1560].

Mucopolysaccharidosis II (Hunter syndrome) may result from large (20–30 kb) deletions or inversions of the *IDS* locus on Xq28. This is believed to result from non-allelic homologous recombination between the functional gene copy and a pseudogene, occurring predominantly during male meiosis [1561].

Duplications including the *MECP2* gene (Xq28), supposedly generated by the FoSTeS mechanism, are sometimes seen in male patients with a specific phenotype, characterised by neurodevelopmental delay, intellectual disability and seizures (*MECP2* duplication syndrome) [1562,1563]. The *MECP2* gene codes for a chromatin-associated protein binding to methylated CpG for activation or repression of transcription of target sequences. Other types of defects in the *MECP2* gene (missense mutations, small deletions causing disruption of the reading frame, nonsense mutations) are associated with a severe neurodevelopmental disorder (Rett syndrome) in heterozygous female carriers [1184]. It is believed that the phenotype of hemizygous males would be so severe that it would cause early intrauterine death. The rare cases of diagnosed Rett syndrome in males are described among individuals with additional X chromosome (47, XXY – Klinefelter syndrome) or somatic mosaics for the mutation [1564]. Alternatively, it has been proposed that at least

some of the affected baby boys were actually born but rapidly succumbed to non-specific congenital onset encephalopathy before getting a diagnosis by molecular methods.

Spinal muscular atrophy (SMA) is a relatively common genetic disease transmitted in autosomal recessive pattern. It is characterised by generalised muscle weakness and atrophy (predominantly proximal), usually starting at early age (although age of onset may actually vary between different grades of severity from infancy to adulthood) and following a progressive course. SMA is associated with mutations in the *SMN1* (survival of motor neuron) gene, encoding a RNA-binding protein required for the assembly of small nuclear ribonucleoprotein complexes. The *SMN1* gene is located within a 500 Kb genomic element on the human 5q chromosome arm (telomeric copy) that is repeated on the 5q in centromeric direction (centromeric copy) [1565]. The telomeric copy (*SMN1*) and the centromeric copy (*SMN2*) of SMN differ by 5 nucleotides only (>99% identity). The *SMN2* gene copy, however, carries a C-to-T transition in exon 7 that alters the splicing pattern of the transcript. The T-allele variant is associated with decreased transcription rate and synthesis of a truncated, dysfunctional SMN protein [1566]. Healthy non-carrier individuals have one telomeric and one centromeric gene copy per haploid genome. The SMA phenotype may arise when both *SMN1* gene copies that an individual has inherited lack exon 7; or the *SMN1* gene copies are lost altogether (sometimes, plus the flanking sequences); or are inactivated (e.g. by point mutations elsewhere in *SMN1*); or are replaced with the *SMN2* sequence (carrying the T allele in exon 7) via gene conversion [1567]. Individuals with deleted or inactivated *SMN1* and 1 or 2 copies of the *SMN2* gene usually exhibit the severe, early-onset from of SMA (SMA type I). Patients carrying 3 or 4 copies of *SMN2* (resulting from inheritance of one or both chromosomes with *SMN1* converted to *SMN2*) usually have the milder, later-onset forms of SMA II and III [1568]. As deficiency of the normal SMN1 protein may be partially compensated by expression of SMN2, multiple compounds have been tested in attempt to stimulate the expression of SMN2 protein and ameliorate the phenotype of SMA. Among these are histone deacetylase inhibitors (e.g. valproic acid, trichostatin A), aclarubicin (an anthracycline derivative), indoprofen (non-steroid anti-inflammatory drug), splicing modifiers, anti-terminators, proteasome inhibitors, inhibitors of signalling pathways, and others [1569-1572]. So far, the clinical trials have not been very successful [1573].

Charcot-Marie-Tooth disease 1A (CMT1A) is a hereditary demyelinating disorder caused by duplication or other type of mutation in the gene encoding the peripheral myelin protein-22 (*PMP22*) [1574,1575]. The duplicated region is within a 1.4-Mb region in 17p12, flanked by two 24-Kb low copy number repeats [1575]. De novo CMT1A duplications arise from unequal crossover during meiosis due to misalignment of the repeated units [1576,1577]. Deletion of the same genomic region is associated with development of another type of inherited neuropathy, hereditary neuropathy with liability to pressure palsies (HNPP) [1578]. Despite the autosomal recessive inheritance of both conditions, the origin of the duplications of *PMP22* in CMT1A and deletions in HNPP has been found predominantly in the paternal germline, although cases of HNPP with maternal inheritance have also been reported [1579,1580].

Cancer, as a microevolutionary process, may also be characterised by 'bursts' of high-level rearrangement activity in the transforming cell, rather than slow accumulation of mutations. Recently, it has been proposed that in some types of tumours (e.g. bone cancer), the genomic instability occurs because of localised fragmentation affecting one or more chromosomes in the genome of transformed cells. The fragments are subsequently 'patched' together by the error-prone mechanism of NHEJ [1528,1581]. The latter creates opportunities for introduction of mutations that may enhance the capacity for proliferation of the cancer cell.

9.4. Swimming in the gene pool without a lifeguard – DNA repair as an evolutionary drive

They told me that the road I took
would lead me to the Sea of Death;
and from halfway along I turned back.
And ever since, all the paths I have roamed
were entangled, and crooked, and forsaken.
Akiko Yosano, from Tangled Hair (1901)

As it was already discussed, 100% precise repair of all alterations in DNA may only be beneficial in the short term, as there would not be opportunity for introduction of changeability in the genome for the sake of adaptation. This would sooner or later bring the population or the species to extinction. There is no specifically designated source of genetic diversity except for meiotic crossover, and it only works on pre-existing genetic variants. The mechanisms of repair, however, are a little 'leaky', allowing for occasional introduction of genomic alteration/s with every generation (every cycle of replication). The 'leakiness' in repair plays a role in the fate of a single cell as strongly as it does in the fate of an organism or a whole species. Thus, (almost) every individual (be it a cell or an organism) produces progeny that is just a little different from its parents. There is, however, no mechanism to decide whether a genetic variant would be fit for independent life and reproduction at the precise moment in time, before it is actually tried out. Therefore, unsuccessful variants may emerge, only to be promptly eliminated from the genetic pool. This goes for individual cells, organisms, populations and species, although the mechanisms may somewhat vary when they work on different targets and in different timescales. Cancer is currently believed to be a microevolutionary process.

Obviously, a sort of compromise must be reached in order to keep the blueprint of genetic information intact and difficult to change so as to protect the health and life of the individual and, at the same time, to ensure that the species would possess just enough genetic adaptability to survive changes in the environment. The capacity for maintaining this fine balance is a continuous process throughout the lifespan of the individual, the time span of the species and the existence of life on Earth. The 'leakiness' of DNA repair ensures that life might change but is unlikely to ever actually end. The random nature of mutagenesis and the relatively low mutation rate in eukaryotic genomes provides not only a clear field for evolution to work without much risk for sudden rapid extinction of populations and species but also a certain 'reserve' of unaltered genomes at any given time. The latter may restore the population or the species whenever a genetic alteration turns out to be deleterious for its carriers for any reason.

There is also the question about the role of error-prone mechanisms of DNA repair in evolution – specifically, NHEJ and translesion DNA copying. The impact of these mechanisms in evolution is quite different. NHEJ might have played a crucial role in eukaryotic evolution. We already discussed that the evolution of NHEJ is markedly accelerated compared to the evolution rates of other genes coding for proteins of repair, possibly because of the role of NHEJ in the establishment of the immune repertoire. It is likely that the error-proneness of NHEJ kept complex living beings healthy throughout evolution by conferring resistance to infectious agents that were deadly to the previous generations. For example, when syphilis was brought to Europe in the XV century, the infected individuals typically died within several months and living into tertiary stage was quite rare. Several centuries later, people that have not received any type of treatment for syphilis only rarely died within a year after infection with syphilis, as the infamous Tuskegee experiment proved. The Tuskegee experiment was, at first, a clinical trial of the incidence of syphilis in the US and effectively became a study on the effects of untreated syphilis on various organs and systems in different races. It began in the early 30-ties of the XX century and was ended because of serious concerns about the ethics of the experiment in 1972 [1582]. Shortly after, in 1974, the National Commission for the Protection of Human Subjects in Biomedical and Behavioral Research of the US was created. Over 400 men participated in the study. The mechanism for enrolment was later found to be non-consensual, that is, the subjects were not informed what they were agreeing to. By the end of the study (about 40 years later), over 70 of the participants in the study were still alive Thus, error-proneness of NHEJ may have been associated with short-term disadvantages (potentially elevated risk for carcinogenesis) but was beneficial in the long term. It has been recently proposed that gain and loss of introns in eukaryotic genes might have occurred as a consequence of repair of double-strand breaks by adding or removing non-template nucleotides to the breakpoint junctions, which is typical for non-homologous end joining [1583,1584].

Translesion replication is also an inherently error-prone mechanism. Effectively, it substitutes one risk for another – instead of the risk of occurrence of a double-strand break in DNA, the risk for incorporation of the wrong nucleotide, altering the sequence of DNA [1585]. As translesion replication ensures temporary survival of damaged cells that are going to die sooner or later anyway, it provides short-term benefits only. Error-prone copying of damaged templates may, therefore, be of importance in microevolutionary processes such as cancer, as it accelerates the rate of accumulation of errors in DNA. Cancer, however, causes elimination of the altered cells from the genetic pool together with the organism; therefore, it is not very likely that translesion replication had played an important role in evolution.

At present, genetic disease in man due to germline and somatic mutations (genetic disease and somatic carcinogenesis) may be viewed as a by-product of ongoing molecular evolution, a kind of collateral damage in Nature's efforts to sustain life on Earth. It is likely that the same may be valid about extinction of populations and species that is not directly related to man-made alterations of the environment. It may seem as a price too high to pay, but there seems to be no other way, as the 3.5 billion years of Earth evolution have proven

so far. This does not mean that the biomedical science should or would ever abandon the efforts to prevent recurrence of genetic disease in affected families or the research of treatment options for the affected individuals. Unlike people, Nature is neither good nor evil. It does not judge its creations, does not favour one over the other, and not reward or punish living things for anything. It simply acknowledges occasional failures and moves on to the next possible option. What we, modern humans, might do, is try to do our best to ensure that everyone, whatever their genetic background, their upbringing, and their beliefs might be, would have a chance to live a fulfilling life, be as comfortable as possible, and contribute to society in their own unique way.

Acknowledgements

This research was supported by Grant No. DFNI-B01/2 at the National Science Fund, Ministry of Education and Science of Republic of Bulgaria.

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